

REMARKS/ARGUMENTS

Specification

The substituted paragraph on page 1 was amended merely to correct the information relating to government funding for research.

Claims

Claims 3-24, 33 are currently pending. Claim 3 has been canceled. Claims 1,2, 25-32, and 34-76 have been withdrawn as directed to a non-elected invention. Claims 77-86 have been added. Claims 4-6, 8-11, 19, and 22 have been amended.

Claims 4, 8, 11, 19, 22, and 33 were amended merely to correct their dependencies. Claims 5 and 6 were amended to recite an isolated polynucleotide "having ABC1 activity". Claims 8-10 were amended to insert the word "pharmaceutically" before the term "suitable carrier", as requested in the Office Action. Support for the claim amendments for 5,6, and 8-10 can be found throughout the specification.

New claims 77-80 are directed to the subject matter of original claim 3, which has been canceled. New claims 81-83 are directed to the subject matter of original claim 11, which originally depended from claim 3 and has been amended in this response. New claims 84-86 are directed to the subject matter of claim 14, which depended from original claim 11. Support for all of these amendments can be found in the original claims as explained above and throughout the specification.

No new matter has been added by way of these amendments.

Claim Objections

Claims 8-10 were amended to insert the word "pharmaceutically" before the term "suitable carrier" to address the objections cited in the Office Action.

Discussion of the 35 U.S.C. § 112 Rejections

Enablement

Claims 3-24 and 33 were rejected under 35 USC § 112, first paragraph, as allegedly not enabled. Applicants respectfully traverse the rejection.

The Office alleges that while the specification is enabling for a polynucleotide comprising SEQ ID NO: 1 (new claim 77); a polynucleotide encoding a polypeptide comprising SEQ ID NO: 2 (new claim 78); and a polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1 (new claim 79), it does not reasonably provide enablement for any polynucleotide comprising a polynucleotide that encodes a polypeptide that is 98% identical to SEQ ID NO: 2 (new claim 80) or any polynucleotide comprising a nucleotide sequence that has either 90% or 95% sequence identity with SEQ ID NO: 1 (claims 5 and 6).

Under 35 U. S. C. § 112, all that is required is that the specification describe the invention in such terms as to enable a person skilled in the art to make and use the invention. Thus, with respect to claim 80, the specification must teach one skilled in the art how to make and use a polynucleotide having ABC1 activity encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2. With respect to claims 5 and 6, the specification must teach one skilled in the art how to make and use a polynucleotide having ABC1 activity comprising a nucleotide sequence that has either 90% or 95% sequence identity with SEQ ID NO: 1. The test for enablement is whether one reasonably skilled in the art (1) could make and use the invention (2) from the disclosures in the patent coupled with information known in the art (3) without undue experimentation. *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988); *United States v. Telectronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988); M.P.E.P. § 2164.01.

Contrary to the Office's allegation, the instant specification provides considerable guidance to enable a skilled artisan to make and use a polynucleotide having ABC1 activity encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2. For example, the specification describes polypeptides having at least 98% sequence identity with SEQ ID NO: 2 at, for example, pages 22, 25, and 31. In addition, the specification describes polypeptide variants and defines the biological activity of a

polypeptide variant at pages 22 and 25. The specification further provides several examples of the types of polypeptide variants and teaches how to design polypeptide variants at pages 23, and 25-28, including teaching the deletion or substitution of non-conserved amino acids as well as conservative amino acid substitution (pages 23 and 27), among others. The specification teaches several methods for identifying and making suitable ABC1 variants at pages 26-28, including structure-function studies and genetic engineering techniques. The specification also teaches that the ABC1 variants can be naturally-occurring or artificially constructed at pages 27 and 28.

Likewise, the instant specification provides considerable guidance to enable a skilled artisan to make and use a polynucleotide having ABC1 activity comprising a nucleotide sequence that has either 90% or 95% sequence identity with SEQ ID NO: 1. For example, the specification describes polynucleotides having at least 90% or 95% sequence identity with SEQ ID NO: 1 at pages 22 and 31. In addition, the specification describes polynucleotide variants and provides examples of polynucleotide variants having biological activity, including those having additions, deletions, and silent substitutions, such as those due to degeneracy of the code at, for example, pages 23, 27, 31 and 39. The instant specification also teaches that the polynucleotides can be produced chemically, enzymatically, or metabolically (specification at pages 19 and 27) and provides a detailed description of the recombinant methods and techniques used to produce the polynucleotides of the invention, including making and amplifying the polynucleotides (specification at, for example, pages 35-40, 43-44, and the Examples). The specification further teaches the sequencing of the polynucleotides and how to determine the percent identity with SEQ ID NO: 1 or a fragment thereof (specification at, for example, 20-22 and 76-79, Example 4). While the recombinant techniques described throughout the specification and in the Examples (particularly Example 4) were used to produce *exemplary polynucleotides* corresponding to SEQ ID NO: 1, these same techniques and other standard recombinant techniques widely known in the art and easily within the skill of the ordinary artisan can be used to produce additional polynucleotides having at least 90% or 95% identity to SEQ ID NO: 1.

Further, the specification provides a detailed description of the methods used to test the claimed polynucleotides for ABC1 activity, including the construction of expression vectors, transfection into host cells and assays for determining cholesterol efflux and ABC1 activity (specification at pages 44-51, 71-74, 79-81 and Examples 1, 6-8).

The specification also teaches one skilled in the art how to use the claimed polynucleotide molecules at, for example, pages 44-61, 67-68, and the Examples section.

The Office alleges that the scope of the claims are not commensurate with the enablement provided by the disclosure because the claims encompass an enormous number of polynucleotide sequences. Further, the Office alleges that while recombinant and mutagenesis techniques are known, it is not routine to screen for substitutions or modifications of nucleotides and given that the results of such changes are unpredictable, the reasonable expectation of success in obtaining the desired activity is limited. Finally, the Office alleges that "changing the nucleotides sequences as proposed by the applicants... may not lead to desired function of the polynucleotides." (Office Action at page 4).

First, given that the present claims are limited to those polynucleotide sequences that have ABC1 activity, they do not encompass an infinite number of polynucleotides as the Office suggests. Second, the specification clearly teaches nucleotide and amino acid substitutions, additions, and deletions that *maintain* the ABC1 activity and teaches to avoid changing nucleotide and/or amino acids that significantly alter the activity of the ABC1 protein. Given that the claims are limited to those polynucleotides encoding a polypeptide having ABC1 activity, variant polynucleotides that do not have ABC1 activity are not within the scope of the present claims.

In addition, as discussed above, the specification teaches one skilled in the art how to produce the polynucleotides of the invention using well-established and standard recombinant techniques that are well within the knowledge and skill of the ordinary artisan. Applicants submit that it is routine experimentation to use recombinant, mutagenesis, amplification, and sequencing techniques to make and determine the sequences of variant polynucleotides. Applicants further submit that it is a matter of routine experimentation to screen variant nucleotide sequences for ABC1 activity using

the techniques described in the specification and other well-known techniques. The law clearly states that "a considerable amount of experimentation is permissible, if it is merely routine." *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Further, the fact that experimentation may be complex does not necessarily make it undue. *Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985); *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Thus, the test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *In re Angstadt*, 537 F.2d 498 (CCPA 1976). Applicants submit that such routine work as producing variant polynucleotides and screening them to determine activity is well within the knowledge and skill of the ordinary artisan and does not involve undue experimentation.

The Office further alleges that the specification does not support the broad scope of the claims because the specification does not establish regions of the polynucleotide sequence that can be modified without affecting activity, the general tolerance of the ABC1 sequence to modification, and a rational and predictable scheme for modifying the sequence with the expectation of obtaining the desired activity. However, with respect to claim 80 (drawn to a polynucleotide encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2), the specification clearly defines and teaches methods for the determination of percent sequence identity at pages 23-28, which methods are routine in the art and within the skill of the ordinary artisan. Further, the specification establishes regions of the polynucleotide sequence that can not be modified without affecting activity by teaching the structural/functional domains relating to ABC1 transporter activity, as well as teaching highly conserved sequences (specification at page 17). The specification additionally teaches examples of specific amino acid substitutions that are not tolerated at pages 28-29 (see SEQ ID NOs: 8 and 10) and 72-74 (Example 1 and corresponding Figure 1) and several amino acid substitutions that are tolerated at page 30.

In addition to the teachings in the specification, the structural/functional domains correlating to its activity as a transporter protein, as well as the regions conserved between different ABC transporter proteins and across species, were characterized and

known in the art at the time of filing the present application. See, for example, Broccado et al, *Biochim. Biophys. Acta*, 1461: 395-404 (1999) and Hayden et al, *Curr. Opin. Lipid.*, 11: 117-122 (2000), both of which are submitted herewith as Exhibit 1. Among other things, Broccado et al. describes sequences conserved between different ABC transporter proteins and across species. Broccado et al., also describes the structural domains in the ABC1 protein and the corresponding polynucleotide domains, as well as provides examples of amino acid residues known to be conserved and others known to be variable. Likewise, Hayden et al, describes the structural domains of ABC1, as well as critical residues that do not tolerate substitution and residues that tolerate substitution. Based on the teachings in the specification, as well as structural information known in the art, a skilled artisan would realize that the nucleotide sequence corresponding to the characterized structural/functional domains would tolerate only limited modification. Further, one skilled in the art would realize that the highly conserved sequences would tolerate limited modification, if any. Further, a skilled artisan would have realized which specific residues could be substituted and which residues could not tolerate substitution.

With respect to claims 5 and 6 (drawn to polynucleotides having 90% and 95% sequence identity with SEQ ID NO: 1, respectively), as discussed above, the specification establishes regions of the polynucleotide sequence that can not be modified without affecting activity by teaching the structural/functional domains relating to ABC1 transporter activity, as well as teaching highly conserved sequences (specification at page 17). In addition, the specification teaches examples of specific nucleotide substitutions that are not tolerated at pages 28-29 (see SEQ ID NOs: 8 and 10) and 72-74 (Example 1 and corresponding Figure 1) and several nucleotide substitutions that are tolerated at page 30. The specification also establishes regions of the polynucleotide sequence that cannot be modified without affecting activity by teaching the sequence and position of transcription regulatory elements in the 5' untranslated region (specification at pages 32-33, Figure 13) as well as sites in the 3' untranslated region (specification at page 33). One skilled in the art would realize that these sites, as well as other known and published sites in the 5' and 3' untranslated regions, would tolerate limited modification, if any. In

addition, the ABC1 activity of sequence variants can easily be tested using the methods described in the specification as well as other routine methods known in the art.

For the reasons discussed above, the claims are fully enabled by the specification. Accordingly, the Applicants respectfully requests withdrawal of the 35 U.S.C. § 112 rejections.

Written Description

Claims 3-24 and 33 were rejected under 35 USC § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor(s) had possession of the claimed invention at the time the application was filed. Applicants respectfully traverse the rejection.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art could reasonably conclude that the inventor had possession of the claimed invention. *See, e.g., id.* at 1116; M.P.E.P. § 2163(I). There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. M.P.E.P. § 2163(I)(A) (citing *In re Wertheim*, 541 F.2d 257, 263, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976)). Thus, a description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. *See, e.g., In re Marzocchi*, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971); M.P.E.P. § 2163.04. Therefore, the Office must have a reasonable basis to challenge the adequacy of the written description and has the initial burden of presenting, by a preponderance of the evidence, why a person skilled in the art would not recognize in an Applicant's disclosure a description of the invention defined by the claims. *See, e.g., In re Wertheim*, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976); *Ex parte Sorenson*, 3 USPQ2d 1462 (Bd. Pat. App. & Int. 1987); M.P.E.P. § 2163.04.

Whether the specification shows that an applicant was in possession of the claimed invention is a factual determination. M.P.E.P. § 2163(I). Possession is shown "by describing the claimed invention with all of its limitations using such descriptive

means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention.” M.P.E.P. § 2163.02 (citing *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572 (Fed. Cir. 1997)). Factors to be considered in determining whether there is sufficient evidence of possession include: (1) the level of skill and knowledge in the art; (2) partial structure; (3) physical and/or chemical properties; (4) functional characteristics alone or coupled with a known or disclosed correlation between structure and function; (5) and the method of making the claimed invention. *Id.* at (II)(A)(2)-(3)(a). Disclosure of *any* combination of such identifying characteristics that distinguish the claimed invention such that one skilled in the art would conclude that the applicant was in possession of the claimed species is sufficient to satisfy written description. *Id.*; see *Reagents of the Univ. of Calif. v. Eli Lilly*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406. Correspondingly, as the Patent Office’s internal guidelines assert, “the written description requirement may be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function.” M.P.E.P. § 2163(II)(A)(3)(a)(i)(C)(2). Thus, when “knowledge and level of skill in the art is high, a written description question *should not be raised* for original claims even if the specification discloses only a method of making the invention and the function of the invention.” *Id.* (emphasis added), see *In re Hayes Microcomputer Products, Inc. Patent Litigation*, 982 F.2d 1527, 1534-35, 25 U.S.P.Q.2d 1241, 1246 (Fed. Cir. 1992) (“An applicant’s disclosure obligation varies according to the art to which the invention pertains.”).

Moreover, that which is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. M.P.E.P. § 2163 (citing *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986)). Further, the written description requirement does not require an actual reduction to practice. M.P.E.P. § 2163. Accordingly, an Applicants need not show that the invention will work for its intended purpose to satisfy the written description requirement.

The Office alleges that the specification does not contain any disclosure of the function of all DNA sequences that are 90% or 95% identical to SEQ ID NO: 1 or the function of polypeptide that is 98% identical to SEQ ID NO: 2. Applicants have

amended the claims to recite that the variant polynucleotide sequences have ABC1 activity. Accordingly, the function of all of the presently claimed polynucleotides is ABC1 transporter activity.

In making its rejection, the Office seems to allege that an actual reduction to practice is required to meet the written description requirement. However, as discussed above, an actual reduction to practice is not the standard for written requirement. Instead, the specification need only describe a combination of identifying characteristics, such as partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention.

In this regard, the specification thoroughly describes the partial structure of the claimed polynucleotides. For example, the specification clearly provides the structure (sequence) of SEQ ID NO:1 in providing the 10,442 bp nucleotide sequence of SEQ ID NO: 1 and the structure (sequence) of SEQ ID NO:1 in providing the 2261 amino acid sequence of SEQ ID NO:2. The specification also clearly describes and provides several examples of variant polynucleotide sequences of SEQ IF NO: 1 and SEQ ID NO: 2, as discussed in detail above (specification at, for example, pages 22-28, 31, and 39).

In addition, the specification clearly teaches the ABC1 transporter function of the claimed polynucleotides, provides demonstration of ABC1 transporter activity in Examples 1, 6-8, and teaches sequences to maintain ABC1 transporter activity at, for example, pages 26-30, 32-33, and 72-74, Example 1 and corresponding Figure 1, Figure 13.

In addition to teaching the structure and function of the claimed polynucleotides, the specification also contains a thorough description of how to make and test the claimed variant polynucleotides for ABC1 activity. For example, the specification teaches how to make the claimed polynucleotides throughout the specification and particularly at pages 26-28 (structure-function studies and genetic engineering techniques) and pages 35-40, 43-44, and the Examples (recombinant methods and techniques). The specification further teaches how to test the claimed polynucleotides for ABC1 activity at pages 44-51, 71-74, 79-81 and Examples 1, 6-8. Using these techniques and other techniques well-

known in the art, one skilled in the art could easily make and test the ABC1 activity of variant polynucleotides having 90% or 95% identity to SEQ ID NO: 1 or variant polynucleotides encoding a polypeptide having 98% identity to SEQ ID NO: 2.

Finally, the specification teaches a correlation between ABC1 sequence and activity by teaching the structural/functional domains relating to ABC1 transporter activity, as well as teaching highly conserved sequences (specification at page 17). The specification also teaches examples of specific amino acid substitutions that are not tolerated at pages 28-29 (see SEQ ID NOs: 8 and 10) and 72-74 (Example 1 and corresponding Figure 1) and several amino acid substitutions that are tolerated at page 30. The specification also teaches a correlation between ABC1 sequence and activity by teaching the sequence and position of transcription regulatory elements in the 5' untranslated region (specification at pages 32-33, Figure 13) as well as regulatory sites in the 3' untranslated region (specification at page 33).

Based on the teachings in the specification of the structure of the claimed polynucleotides, its functional characteristics, the correlation between its structure and function, and the methods of making the claimed polynucleotide molecules, as outlined above, Applicants have clearly described identifying characteristics that distinguish the claimed polynucleotide molecules, such that one skilled in the art would conclude that Applicants were in possession of the claimed invention.

For the reasons discussed above, the claims satisfy the written description requirement. Accordingly, the Applicants respectfully requests withdrawal of the 35 U.S.C. § 112 rejections.

Discussion of the 35 U.S.C. § 102/103 Rejections

35 U.S.C. § 102(b)/103(a) Rejection in view of Langmann et al.

The Office rejected claims 3-24 and 33 under 35 U.S.C. § 102(b) as allegedly being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over Langmann et al (Swiss Protein Accession No. O95477; GenBank Accession No. AJ012376 and BBRC, April 1999, 257(1):29-33). The rejection is respectfully traversed.

New claims 77-80 correspond to the subject matter of original claim 3. Claim 77 is directed to an isolated polynucleotide comprising SEQ ID NO: 1. Claim 78 is directed to an isolated polynucleotide encoding a polypeptide comprising SEQ ID NO: 2. Claim 79 is directed to an isolated polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1. Claim 80 is directed to an isolated polynucleotide encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2.

Under 35 U.S.C. § 102(b), a claim is anticipated only if each and every element as set forth in the claim is found in a single art reference. *Verdegaal Bros. v. Union Oil Co.*, 814 F.2d 628, 631, 2 USPQ2d 1051, 10533 (Fed. Cir. 1987); *In re Recombinant DNA Technology Patent and Contract Litigation*, 30 USPQ2d 1881 (S.D. Ind.1993) ("A patent is anticipated only if all the elements and limitations of the claims are found within a single, prior art reference."); *Structural Rubber Products Co. v. Park Rubber Co.*, 749 F.2d 707, 716 (Fed. Cir. 1984) (All elements of the claimed invention must be contained in a single prior art disclosure and must be arranged in the prior art disclosure as in the claimed invention); M.P.E.P. § 2131. Furthermore, no difference may exist between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of invention. *In re Recombinant DNA Technology Patent and Contract Litigation*, 30 USPQ2d 1881 (S.D. Ind.1993). Also, the identical invention must be described or shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989); *Chester v. Miller*, 15 USPQ2d 1333 (Fed. Cir. 1990); M.P.E.P. § 2131.

The Office alleges that Langmann et al. anticipates or obviates claim 3 and the dependent claims because it discloses a polynucleotide that encodes a polypeptide which is more than 98% identical to SEQ ID NO: 2. Applicants appreciate the facsimile copy of the Blast Sequence Alignment Report that the Examiner forwarded on August 29, 2003. However, Applicants respectfully submit that the incorrect sequence was used for the sequence alignment generated by this report. Specifically, Applicants submit that the amino acid sequence used to generate the sequence alignment is not the amino acid sequence taught by Langmann et al. The first twenty amino acids of the sequence disclosed in the Langmann reference (BBRC, April 1999, 257(1):29-33), GenBank

Accession No. AJ012376, and Swiss Protein 095477 are: MPSAGTLPWVQGIICNANNP (see attached Gen Bank report for AJ012376, which references BBRC, April 1999, 257(1):29-33 and Swiss Protein: 095477; Exhibit 2). However, the first 20 amino acids for the sequence used in the report are: MACWPQLRLLLWKNLTFRRR (see page 3 of the report attached herewith; Exhibit 2).

The Office rejects claim 3 apparently for reciting a polynucleotide that encodes a polypeptide which is more than 98% identical to SEQ ID NO: 2 and does not address the other polynucleotides listed in claim 3. Despite the lack of reasoned rejection for the other polynucleotides recited in original claim 3, Applicants provide the following brief comments.

The sequence disclosed in Langmann et al. (and the corresponding Swiss Protein Accession No. 095477 and GenBank Accession No. AJ012376) does not anticipate claims 77-80. Langmann et al. discloses an ABC1 polypeptide of **2201 amino acids** and a corresponding **6880 bp polynucleotide** having a **6603 bp open reading frame**. In contrast, claim 77 is directed to an isolated polynucleotide having ABC1 activity comprising SEQ ID NO: 1, which is a **10,442 bp polynucleotide** having a **6783 bp open reading frame**. Given that Langmann et al. teaches a polynucleotide having only 6880 nucleotides, it does not anticipate claim 77. Further, the disclosure of Langmann et al. does not render claim 77 obvious. Langmann only teaches a 6783 bp polynucleotide that does not even include the entire coding region of ABC1. As taught in the instant application, the additional nucleotides found in SEQ ID NO: 1 constitute sequences located at the 5' end of the coding region, as well as sequences in the 5' and 3' untranslated regions. As provided in the specification at, e.g., pages 32-33 and Examples 17 and 18, these 5' and 3' end sequences contain regulatory sequences of the ABC1 gene. As known in the art, general regulatory sequences in the 5' untranslated region are critical for initiation of transcription (i.e., SP1 sites and TATA box) and initiation of translation of ABC1. Further, as taught in the specification at pages 88-95 and Figure 13, the 5' untranslated region also contains binding sites for transcription factors with roles in lipid metabolism (i.e., LXR and sterol response elements). Thus, the newly described 5' nucleotides found in SEQ ID NO: 1 not only contain coding region sequences, but further

contain gene-specific regulatory sequences directly involved in the regulation of ABC1 gene expression and cholesterol transport. In addition, as known generally in the art and further taught in the specification at page 33, the sequences in the 3' untranslated region contain sequences important for transcript stability. Given that the newly described 5' and 3' end regulatory sequences found in SEQ ID NO: 1 affect the level and stability of the ABC1 transcript, and consequently the level of ABC1 protein, they play an important functional role in cholesterol transport. These sequences are not disclosed in the Langmann et al. reference; indeed, the Langmann et al. reference would lead one of ordinary skill to believe that such sequences were *not* a part of the ABC1 cDNA. Accordingly, Langmann et al. does not obviate the polynucleotide recited in claim 77.

Nor does Langmann et al. anticipate or obviate claims 5 and 6, which are directed to an isolated polynucleotide having ABC1 activity comprising a nucleotide sequence that has at least 90% identity or 95% identity with the polynucleotide comprising SEQ ID NO: 1, respectively. Assuming 100% nucleotide sequence identity between the 6880 bp polynucleotide disclosed by Langmann et al. and SEQ ID NO: 1, a polynucleotide sequence that is 6880 bp has, at best, only 66% identity with SEQ ID NO: 1, which is clearly outside the scope of the claims.

Claim 78 is directed to a polynucleotide encoding a polypeptide comprising **2261 amino acids** (SEQ ID NO: 2). Given that Langmann et al. teaches a polypeptide having only **2201 amino acids** (i.e., lacking 60 amino acid residues found at the N-terminus of SEQ ID NO: 2), it does not anticipate claim 78. Further, the disclosure of Langmann et al. does not render claim 78 obvious. Langmann et al. clearly teaches an ABC1 protein having only 2201 amino acids and does not even contemplate an ABC1 protein having a greater number of amino acids, i.e., 2261 amino acids. In fact, by teaching the incorrect methionine start site and by teaching a 2201 amino acid ABC1 protein, the Langmann et al. reference teaches away from the presently claimed polynucleotide encoding a polypeptide of **2261 amino acids** (SEQ ID NO: 2).

Claim 79 is directed to an isolated polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1. The sequence containing nucleotides 291-7074 of SEQ ID NO: 1 corresponds to the full-length open reading frame sequence of ABC1 (6783 bp).

Langmann discloses a polynucleotide sequence having an open reading frame of only 6603 bp. Due to the failure to recognize that the open reading frame is actually 6783 bp in length, the Langmann et al. reference fails to identify 180 bp of the 5' end of the open reading frame and does not even disclose the terminal 60 bp located at the 5' end of the open reading frame. Accordingly, the sequence disclosed in Langmann et al and GenBank Accession No.AJ012376 does not disclose a polynucleotide sequence that contains nucleotides 291-7074 and thus fails to anticipate claim 79. Further, the disclosure of Langmann et al. does not render claim 79 obvious. Langmann et al. clearly teaches a polynucleotide sequence having an open reading frame of only 6603 bp and does not even contemplate a polynucleotide sequence having a longer open reading frame, i.e., 6783 bp. In fact, by teaching the incorrect open reading frame and by teaching an open reading frame of 6603 bp, the Langmann et al. reference teaches away from the presently claimed polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1.

Claim 80 is directed to an isolated polynucleotide encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2. The polypeptide disclosed in Langmann et al. has 2201 amino acids. The polypeptide of SEQ ID NO: 2 has 2261 amino acids. Assuming 100% amino acid sequence identity, a polypeptide sequence that is 2201 amino acids in length has, at best, only 97% identity with SEQ ID NO: 2. Further, there are at least 7 amino acid differences between the Langmann et al. sequence and SEQ ID NO: 2 as taught in the specification at page 30. Thus, the 2201 amino acid polypeptide disclosed in Langmann et al. is not a polypeptide having at least 98% sequence identity with SEQ ID NO: 2, as required by claim 80. Given that the 2201 amino acid sequence taught in Langmann et al. does not teach each and every element as set forth in the claim, it does not anticipate claim 80. Further, given that Langmann et al. clearly teaches an ABC1 polypeptide having only 2201 amino acids and does not even contemplate an ABC1 polypeptide having a greater number of amino acids, i.e., 2261 amino acids, Langmann et al. does not teach or suggest the polypeptide of SEQ ID NO: 2 (i.e., polypeptide having 2262 amino acids); nor does it teach or suggest a polypeptide having at least 98% sequence identity with SEQ ID NO: 2.

The Office further alleges that the dependent claims are obvious over Langmann et al. However, given that the disclosure of Langmann et al. does not render claims 77-80 (i.e., corresponding to original claims 3) or claims 5 and 6 obvious for the reasons stated herein, Langmann et al. does not render obvious the claims dependent thereon.

Applicants respectfully request withdrawal of the 102(b)/103(a) rejection based on Langmann et al. and GenBank Accession No. AJ012376.

35 U.S.C. § 102(e)/103(a) Rejection in view of Rosier-Montus et al.

The Office rejected claims 3-24 and 33 under 35 U.S.C. § 102(e) as allegedly being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over Rosier-Montus et al. (US 20020146792A1). The rejection is respectfully traversed.

Rosier-Montus et al. was published on October 10, 2002, with a priority date of May 2, 2000. Applicants submit herewith an Inventor Declaration under 37 CFR §1.131 to establish a date of invention prior to the May 2, 2000 priority date of Rosier-Montus. See Exhibit 3, Declaration of Dr. Richard Lawn, and the supporting Exhibits A-D. Given that the inventions claimed in the present application were invented prior to May 2, 2000, Rosier-Montus is not applicable prior art.

Accordingly, Applicants respectfully request withdrawal of the 35 U.S.C. § 102(e)/103(a) rejections in view of the Rosier-Montus reference.

Conclusion

In view of the above remarks, the application is considered to be in good and proper form for allowance and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of this application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,
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Review

The ABCA subclass of mammalian transporters

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Abstract

We describe here a subclass of mammalian ABC transporters, the ABCA subfamily. This is a unique group that, in contrast to any other human ABC transporters, lacks a structural counterpart in yeast. The structural hallmark of the ABCA subfamily is the presence of a stretch of hydrophobic amino acids thought to span the membrane within the putative regulatory (R) domain. As for today, four ABCA transporters have been fully characterised but 11 ABCA-encoding genes have been identified. ABCA-specific motifs in the nucleotide binding folds can be detected when analysing the conserved sequences among the different members. These motifs may reveal functional constraints exclusive to this group of ABC transporters. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: ABCA subclass; ABC1; ABCR; Consensus motif; Gene family

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1. Introduction

The family of ABC transporters is one of the largest family of proteins. In genomes whose sequence has been completed they represent between 2–5% of the whole coding regions [1–4]. An arbitrary extrapolation to the human genome which is estimated to contain 60–70 000 genes, will lead to an expected number of ABC transporters of more than 1000, which is likely to be by far an overestimation. Since a comprehensive list is present in the ABC transporter web page (<http://www.med.rug.nl/mdl/human/abc.htm>), and the ABC transporters in various organisms are reviewed in detail in this special issue, we concentrate here only on the ABCA subfamily.

The structural archetype of a full size ABC transporters encoded by a single gene is the P-glycoprotein which possesses a canonical 6×2 transmembrane spanners paired in tandem to the nucleotide binding domains (NBD). However, several subfamilies can be identified on the basis of structural features. Our aim here is to discuss the distinctive structural features of the ABCA subfamily, which appears to be restricted to multicellular organisms. Indeed, the class of mammalian ABC1-like proteins, or, according to the novel nomenclature proposed by the genome project ABCA proteins, lacks a yeast structural ortholog [5–7]. Interestingly, however, transporters sharing similar features can be found in other evolutionarily distant organisms (see below).

2. Features of the ABCA class of ABC transporters

When analysing the prototype of this class, ABCA1 (formerly ABC1), we reported the presence of a regulatory domain reminiscent of the R domain in CFTR. However, here this domain is split into two halves by a highly hydrophobic segment, which we called HH1 [8]. Based on secondary structure predictions, which predicted HH1 as a transmembrane segment but favoured a β conformation, and from analogy to similarly structured domains in K^+ channels [8–11], we proposed the topological model shown in Fig. 1, panel A. The experimental support to this model comes from the analysis of the protease sensitivity of a number of Myc-tagged ABC1 chimeras, transcribed and translated in vitro in the pres-

ence of microsomal membranes (Hamon and Chimini, unpublished) and immunoprecipitated with the anti-myc monoclonal antibody 9E10 [12]. The validity of the model was also examined by a specific antibody reacting with the first NBD of ABC1 [13]. These experiments allowed to define the position of the extracellular loop between TM5 and TM6, which contains a glycosylated asparagine, to confirm the intracellular location of the two ABC domains, as well as the cytosolic location of the second half of the regulatory domain. However, as it is frequently the case with polytopic membrane proteins, alternative topologies cannot be excluded. An example is shown in Fig. 1, panel B, where the HH1 domain is represented as spanning the membrane once from inside to outside. This prediction includes a large extracellular loop of 260 residues, corresponding in the former model to the second half of the R domain. We are currently testing these models by analyzing the reactivity of surface-exposed transporters with a panel of antisera, generated against predicted extracellular loops. For the following discussion we will refer to the topology model shown in panel A.

3. The ABCA gene family

In 1994 we reported the identification of ABCA1 and its structural peculiarities among ABC transporters [8]. It soon became apparent that ABC1 was not a unique example and a large group of transporters shared similar features [8,14–17] (Table 1). Indeed, so far four ABCA genes have been fully sequenced, namely ABCA1, ABCA2, ABCA3 or C and ABCR, either in mouse, man or other mammals. We have recently identified a fifth transporter conserved in mouse and man (tentatively called ABCA7 or ABCX (Broccardo et al. submitted and Dean and Allikments, personal communication). and have evidence for the presence of at least five other individual ABCA genes. Recently, the analysis of osteoclast-specific transcripts has also allowed the identifications of partial sequences (named OCA), closely related to the ABCA family (Wagstaff, personal communication). Their relationship to known ABCA transporters, however, is still unknown.

A pairwise comparison of the published full nucleotide sequences for ABCA cDNAs shows an over-

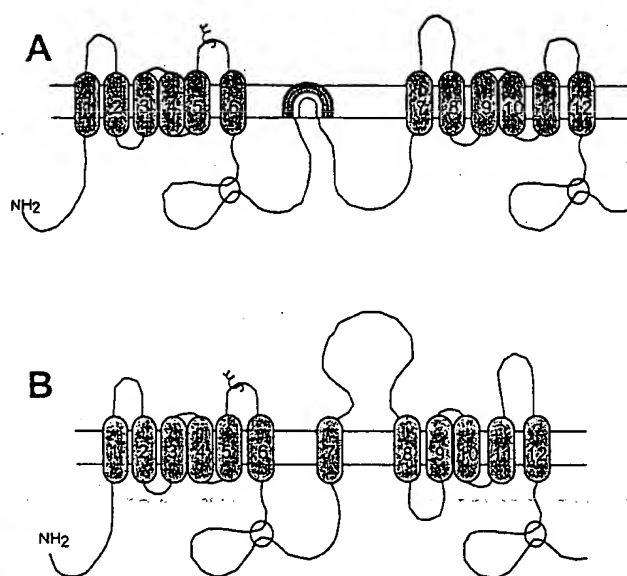


Fig. 1. Predictive topology of ABCA1 as a prototype of the ABCA subclass of transporters. (A) Topological model proposed by the authors with two symmetrical halves interrupted by an R-domain. The highly hydrophobic segment (HH1) is drawn as a hairpin crossing the membrane twice. Each half is composed of a set of six transmembrane spanners and an ATP binding cassette. (B) Alternative topological prediction, with HH1 spanning the membrane once, from the inside to the outside. This leads to an asymmetric structure with a large extracellular loop followed by an odd number of transmembrane spanners in the second half of the transporter.

all identity of around 60% among the different genes, irrespectively of the pairs analysed. Cross-species conservation of individual genes is extremely high, exceeding 85% identity. This allows to design mem-

ber-specific probes, detecting unique transcripts in Northern blots (Fig. 2). Indeed, although the length of each transcript is around 7 kb, slight differences in size can be seen when the RNAs are separated on agarose gels, as shown in panel A. In addition, when a panel of tissues and cell lines is used to hybridise, all these genes appear to be expressed ubiquitously at low levels. Still, a preferential and non-redundant territory of expression can be assigned to individual members. ABCA1 is 10-fold overexpressed in the uterus of pregnant women [8], followed by the expression levels in liver, adrenals and normal uterus, whereas ABCA2 is overexpressed in the adult brain, and ABCA3 in the liver, lung and kidney (Fig. 2). The most recently recognised member, ABCA7 or ABCX, is preferentially expressed in the spleen and more generally in lymphoid organs (not shown, Broccardo et al., in preparation). ABCR was not included in this analysis, since its expression has been reported to be restricted to the retina [18,19].

The currently estimated number of ABCA genes is at least 11, based on the analysis of dbEST by Dean and co-workers (personal communication). A similar estimation was obtained in our laboratory, by a genomic amplification assay with degenerate primers targeting ABCA-specific consensus motifs, which flank conserved introns in the NBDs (see below and Broccardo et al., unpublished observation).

All the fully identified ABCA genes map in syntenic regions in the mouse and human genome, respectively, and there is no evidence of gene clustering (Table 1). This suggests an evolutionary origin of

Table 1
The family of ABCA transporters

Name	Symbol	Chromosome human	Chromosome mouse	RNA	Amino acid	Exons	Accession
ABC1	ABCA1	9q22-q31	4A5-B3	6.9	2259	48	AJ012376
ABC2	ABCA2	9q34	2A2-B	6.7	> 2174	> 42	U18235
ABC3 (ABC-C)	ABCA3	16p13.3	17B	6.5	1704	?	U78735
ABCR	ABCA4	1p22	?	7.3	2273	50	NM000350
ABC4	ABCA7	19p13.3	10B4-C1	6.6	> 1840	> 37	-
EST90625	ABCA5	17q21-q24	?	?	?	38	U66672
EST155051	ABCA6	17q21	?	7	?	38	U66680
KIAA0822	ABCA8	17q24	?	5.7	1581	38	AB020629
EST640918	ABCA9	17q24	?	?	?	38	-
EST698739	ABCA10	17q24	?	?	?	38	-
EST1133530	ABCA11	4p16-pter	?	?	?	?	-

For yet unpublished results the sources are Broccardo et al., (ABCA7) and Dean et al. (ABCA5-6 and 8-11). KIAA0822 [45]

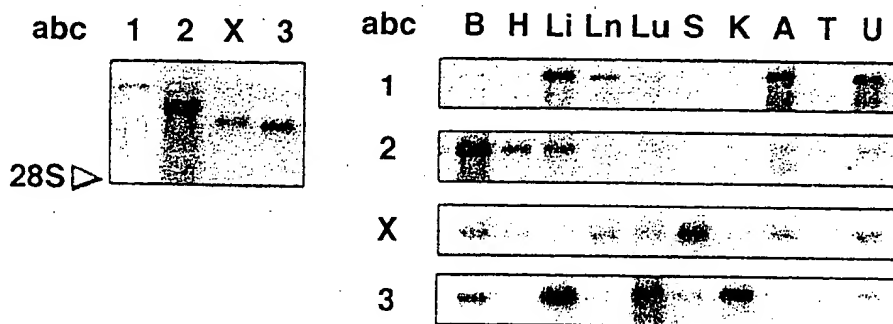


Fig. 2. Expression of ABCA transcripts. (A) Northern blot analysis of brain RNA with ABCA member-specific probes highlights small, but detectable differences in size. ABCA1 transcript is the largest one, followed by ABCA2, ABCA7 and ABCA3. (B) Northern blot analysis of a panel of RNAs from adult mouse tissues, hybridised with member-specific probes shows that all ABCA transcripts are detected ubiquitously at low levels. Non-redundant member-specific territories of preferential expression are nonetheless detectable. 1: ABCA1, 2: ABCA2, X: ABCA7, 3: ABCA3, B: brain, H: heart, Li: liver, Ln: lymph nodes, Lu: lung, K: kidney, A: adrenals, T: thymus, U: uterus.

individual members, predating speciation. Dean and co-workers have recently identified an additional ABCA gene on chromosome 4p16-pter, and a cluster of tandemly linked ABC genes on chromosome 17q24, apparently sharing similarities to the ABCA

class (Dean, personal communication). A more detailed analysis and a final hypothesis about their evolutionary relationships will have to wait for the complete sequences and the mapping of the orthologs in the mouse genome.

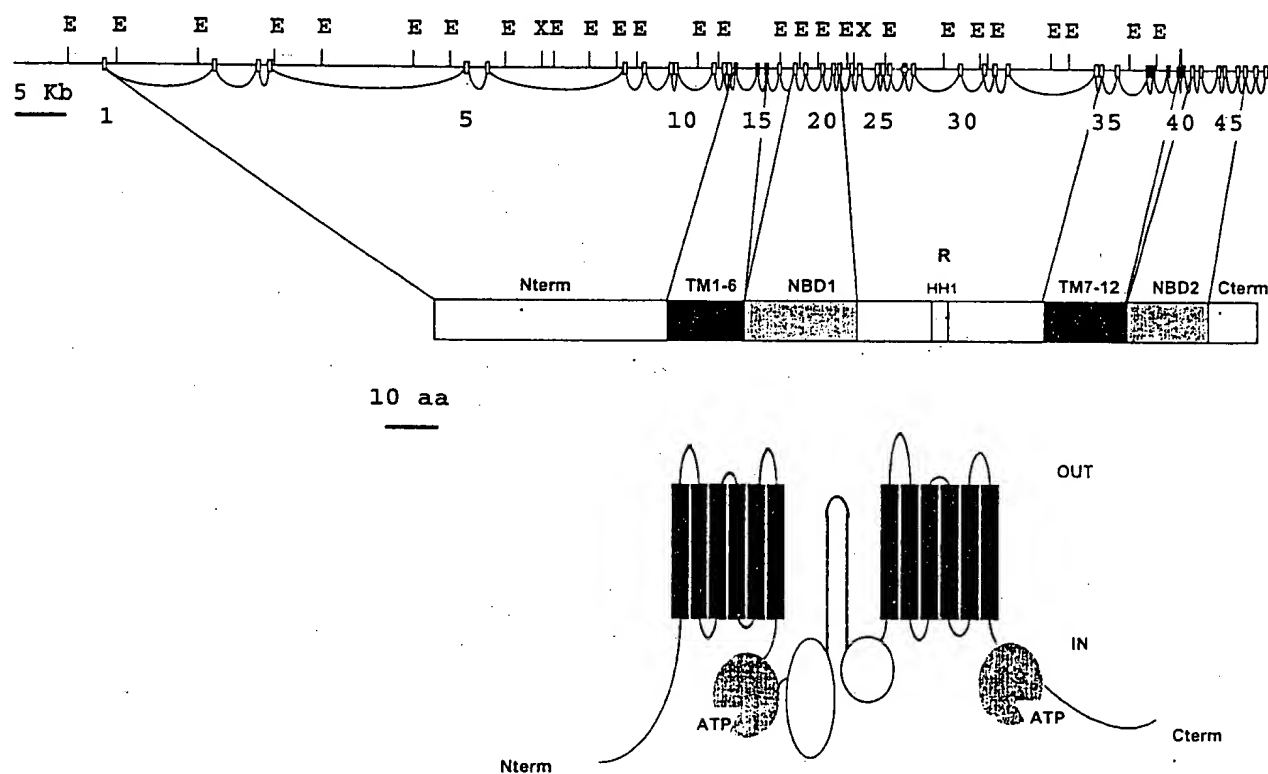


Fig. 3. Correspondence between genomic structure of the ABCA1 gene and protein domains. (Top panel) Exon-intron structure of the ABCA1 gene on mouse chromosome 4. Exons are shown as filled boxes. The positions of *EcoRI* (E) and *XhoI* (X) restriction sites are given. (Middle and bottom panel) Exon-encoded protein domains are highlighted by graphic motifs, either in the linear representation or in the predicted protein. TM: transmembrane helix, NBD: nucleotide binding domain, R: regulatory domain.

4. Gene organisation

A full genomic organisation has been published for human ABCR [20,21]. That of the mouse ABCA1 gene is schematically shown in Fig. 3 and has been deposited in the databases. The mouse ABCA1 gene spans more than 100 kb on chromosome 4A5-B3, and is split into 49 exons (Broccardo et al., personal communication). The overall exon organisation and the correspondence between exons and the encoded protein domains are shown in Fig. 3. It has to be noted that a striking conservation of the gene structure organisation is present among the different members of the ABCA subclass. Most of the splice junctions are indeed perfectly conserved among the different genes, for instance 42 out of the 48 introns of ABCA1 are perfectly conserved in ABCR, both in position and type [20]. No clusters of introns of the same type can be evidenced, however, in any of the analysed genes.

A cross-gene analysis of the intron-exon organisation reveals the highest conservation among members in the regions encoding the NBDs and the transmembrane anchors (Fig. 4 and 5). In contrast, a comparison of the structure of the N- and C-terminal halves of a single gene shows that equivalent domains do

not share a similar genomic organisation. This suggests an independent evolution, which is not unusual among ABC transporters. It does once again corroborate the evolutionary hypothesis of the origin of multidomain transporters from fusion of independently evolved individual domains, as opposed to internal duplication events giving rise to an ancestral transporter. A comparison of the structural organisation of the ABCA gene subclass with other structural classes, like MDR, MRP, CFTR or SUR transporters, shows class-specific diversifications and, again, corroborates the autonomous evolutionary history of each class of genes [22–26].

Finally, and in contrast to the NBD and TD encoding regions, the genomic fragment encoding the regulatory domain shows an extreme variability in the positions of splicing sites among individual transporters and in the number of exons (10 exons in ABCA2 and ABCA7, 13 for ABCA1 and ABCR). This variability is reflected down to the amino acid level, since this domain shows the least conservation in primary sequence. Its main characteristics, however, like the richness in charged residues, the presence of several putative phosphorylation sites, and the presence of the highly hydrophobic spanner, are constantly present. In spite of the invariable detec-

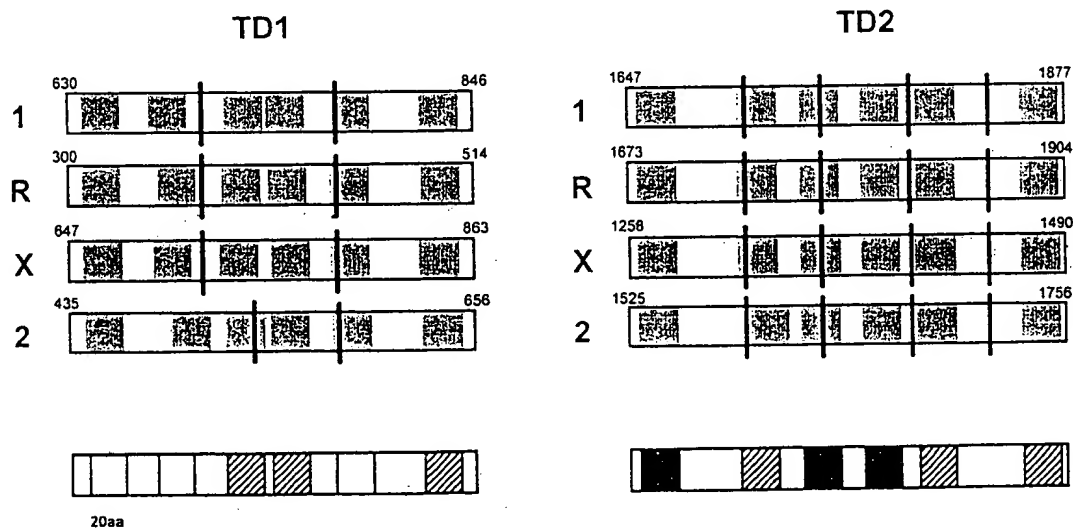


Fig. 4. Conservation of the gene structure across four different ABCA transporters. Transmembrane Domains (TD1 = N-terminal, TD2 = C-terminal). The amino acid positions, taken as borders, are indicated. For simplicity, the limit of each domain corresponds to the limits of the exons. The predicted TM spanners are shown in grey. The bottom rectangle shows the percentage of sequence identity across members for each spanner. Symbols: black solid square: 40–60%, thick hatched squares: 20–40%, thin hatched squares: 0–20% of identity. Exon-introns boundaries are shown by vertical bars. 1: ABCA1, 2: ABCA2, X: ABCA7, R: ABCR.

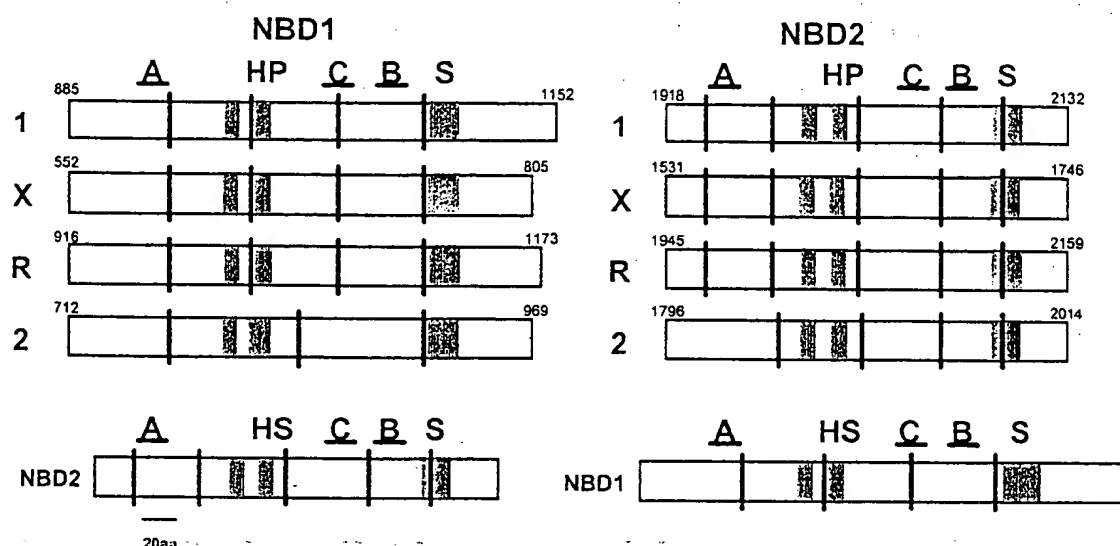


Fig. 5. Conservation of the gene structure across four different ABCA transporters. (NBD1 = N-terminal, NBD2 = C-terminal). The comparative genomic structures of four ABCA transporters are shown. The boundaries of the domains are indicated and correspond to the exons of interest. Introns are indicated by black bars. Conserved motifs are indicated by shaded bars. HS: hot spot, S: switch region, A: Walker A, B: Walker B, C: active transport signature. The bottom rectangle shows for comparison the organisation of a typical ABCA NBD2 or NBD1. 1: ABCA1, 2: ABCA2, X: ABCA7, R: ABCR.

tion of HH1, its position varies largely in each member; this leads to member-specific configurations of the R domain.

5. Sequence conservation and ABCA signatures

At the amino acid level, the known ABCA transporters show an identity from 45–66% along the whole sequence. The comparative analysis of their hydrophobicity plots also reveals highly similar profiles, leading to close, but unfortunately, still ambiguous predictions of topology.

For the sake of simplicity, the following discussion on ABCA sequence conservation will be articulated according to a conventional segmentation in do-

main. The border of each domain has been fixed according to exon limits. We define TD as the group of transmembrane helices and their intervening loops. This corresponds in ABCA1 to residues 630–846 for the N terminal TM 1–6 (exon 14–16) and from 1647–1877 for the C terminal set of membrane spanners (TM 7–12; exon 36–40). In the figure presented we label these limits for each of the analysed transporters.

An analysis of the sequences of TDs across the individual ABCAs demonstrates a higher degree of conservation in the second set of spanners (illustrated schematically in Fig. 4), in spite of a relatively low degree of primary sequence conservation, compared to that in the NBDs (average identity score of 44% for TD1 and 53% for TD2). This is particularly strik-

Table 2
ABCA specific signatures can be highlighted in the extended NBDs

	Walker A - #1	#50	Hot spot	ATS/C	Walker B	Switch #162
NBD1	GQ---LGHNAGKTTT	G-CPQ•N	LTV•EH•FY	LSGGM•RK	LDEPT•G•DP	L•TH•MDEA•LGDR
NBD2	GECFGLLVNGAGKSTT	GYCPQFD	LTGRE•L	YSGG•KRK	LDEPTTGMDP	LTSHSMEECEALC•R

For simplicity the position of Walker A is numbered as +1. Residues in bold are conserved in all subclasses of the ABC transporter family. Normal face residues are conserved at 80% in ABCAs, whereas • corresponds to variable residues. NBD1 or NBD2 corresponds to the N or C-terminal NBD.

ing for the TM VII, IX and X, which all show identities higher than 50%. In addition, according to model A, the first and last extracellular loops in both sets of transmembrane regions show a divergent behaviour in term of sequence conservation. Whereas the 21 amino acid long loop between transmembrane helices I–II and the 36 amino acid long loop between TM VII–VIII are extremely conserved, the last extracellular loop (V–VI; 26 amino acids and XI–XII; 32 amino acids) can be considered hypervariable among the individual transporters.

The NBD corresponds to the extended nucleotide binding domain, i.e. in ABCA1 it spans from amino acid 885–1152 for the N-terminal one (NBD1: exon 18–22) and 1918–2132 for the C-terminal one (NBD2, exon 42–47). An overall analysis of sequence conservation at the NBDs shows an identity from 55 to 61% (average 59%) for NBD1, which increases to 57–69% (average 65%) at the NBD2. As already noted for the exon organisation, an intramolecular comparison of the two NBDs leads to a lesser degree of conservation, with an average value of 34.4%. This is definitely higher than the identity score between corresponding NBDs in ABC transporters belonging to different structural classes. These values range from 21 to 26% when comparing CFTR, MDR or TAP NBDs with the corresponding domains in ABCA1. A comparison of the two NBDs in other ABCs leads to variable results. For instance, in the case of CFTR, the identity score is 24%, while for MDRs and the two TAPS the score is 57 or 55%, respectively.

A closer analysis of NBD conservation leads to the definition of motifs unique to the ABCA family. These are summarised in Table 2 and detailed below. As required to belong to the family of ABC transporters, all ABCA proteins show in the NBDs the classical consensus Walker A, B and C, or ATS motifs (see [27]). Nonetheless, as it is the case for the different structural subgroups in yeast, specific signatures can be highlighted. First, at the level of the Walker motifs, a high conservation of residues surrounding either the conserved glycines in Walker A or the aspartate in Walker B can be documented. It is of note that similar conservations are found both in NBD1 and NBD2.

At least three other ABCA motifs can be defined. The so called 'switch' region, around the key histi-

dine residue, downstream to Walker B, is highly conserved. Similarly, the cluster of residues surrounding the glutamine +50 from Walker A, and the 'hot spot' region, that roughly corresponds to the position of DF508 in CFTR [6,27,28] are conserved. In ABCAs the histidine is close to a highly acidic sequence, whereas the glutamine is invariably preceded by the doublet cysteine-proline. In the MDR/TAP subfamily there is no evidence for an acidic stretch of residues nearby the histidine, whereas both the histidine and the charged stretch are lacking in the CFTR/MRP cluster. All these consensus motifs are detectable in both NBDs, in spite of minor differences. An additional characteristic of the ABCA proteins is the symmetrical presence, downstream to both NBDs (+100–130 amino acids from Walker B), a conserved stretch of amino acids (T • EE•FL • V•E for the NBD1 and D•SV•Q• •L E/D N/Q VF), whose significance is so far unknown.

6. ABCAs in invertebrates

In order to estimate the reliability of the above described motifs, we scanned the ABC sequences in the databases from different genomes. This resulted in the identification of potential ABCA transporters in plants, insects and lower organisms. For instance, in *Arabidopsis thaliana*, several sequences encoding partial ABCA transporters could be detected. After eliminating redundancy and overlapping clones, we concluded that at least two full size ABCA sequences have been already sequenced in *Arabidopsis*, namely AC002339 in chromosome II, and AL049746 on chromosome III. Similarly, in *Dictyostelium discoideum*, the *abcA* sequence (U66526) shows high similarity to the ABCA subfamily, and again, all the above described motifs are present. In contrast to the mammalian ABCAs, however, *AbcA* possesses a unique NBD. Three ABCA full size transporters can be found in the *Caenorhabditis elegans* genome. One corresponds to *ced-7* [29], which can be considered the functional ortholog of ABC1 in the nematode (see below), the two others share an identity higher than 40% at the amino acid level and correspond to AF10131 and AF003146 [30]. From a scan of the *Drosophila* genome project, three genomic sequences, potentially translating into ABCA trans-

porters with a full-size multidomain structure, were detected (AF034856, AC004321, AC004348). Preliminary information from F. Gamarro (personal communication) indicates that ABCA-encoding sequences, identifiable by the presence of these diagnostic motifs, exist also in parasites like *Trypanosoma cruzi* and *Leishmania tropica*.

7. ABCA1 and ced-7: the engulfment of apoptotic corpses

Among the members of the ABCA subgroup of transporters, ABCA1 has been functionally associated to the process of the engulfment of cells dying by apoptosis [13]. Several lines of evidence support the hypothesis. The first derives from the analysis of the ABCA1 expression pattern during mouse embryonic development. This analysis showed a direct spatio-temporal relationship between the expression of ABCA1 transcript and the areas of programmed cell death. In these areas the locally recruited macrophages, known to express ABC1, are actively engaged in the clearance of cell corpses. Consistently, in an 'in vitro' situation, the inhibition of ABCA1 function by a specific antibody greatly reduces the ability of peritoneal macrophages to phagocytose apoptotic thymocytes. This antibody has no effect on their ingestion of yeast, thus functionally linking ABC1 to the process of apoptotic engulfment. Last but not least comes the recent demonstration that an ABCA transporter of *C. elegans*, sharing around 30% identity with ABCA1, is able to complement ced-7 mutants [29]. Ced-7 belongs to one of the two epistatic groups of genes controlling the clearance of corpses generated by cell death during the development of the nematode. Altogether, these results strongly suggest that ABC1 and ced-7 are orthologs in these two evolutionary distant species.

It has to be noted, however, that although the similarities between ced-7 and ABC1 are quite high, in the absence of functional data it would have been extremely difficult to select the best candidate as an ortholog among the mammalian members of the ABCA class of transporters. Indeed, an exclusive analysis of sequence comparisons reveals roughly equivalent identities, ranging from 27 to 32% between ced-7 and each of the individual ABCA trans-

porters. The precise molecular function exerted by an ABC transporter during the engulfment is, however, still an open question. Recent and preliminary results suggest an involvement of ABCA1 in the control of membrane lipid composition. Indeed, both in ABCA1 null mice and in overexpressing transfectants, an altered membrane mobility of phospholipids can be assessed (Broccardo et al., submitted). This might well fit the recently proposed concept that the engulfment of apoptotic bodies is an exquisite form of phagocytosis, since it requires the transbilayer movements of lipids on the surface of the phagocytes (Marguet et al., in press). Along the same line, mutations in the human gene encoding ABCA1 have been detected in patients affected by Tangier disease [31–34], a rare autosomal recessive disorder of lipid metabolism [35,36] whose locus indeed has been mapped to human chromosome 9q31 [37].

8. ABCR and chorioretinal degeneration

Frequently ABC transporters have been associated to diseases. In the group of ABCAs, so far only ABCR has been associated unambiguously to the pathogenesis of degenerative eye diseases [38–40]. In fact it has been reported by several groups that mutations in the ABCR coding regions can be detected in a large panel of degenerative illnesses of the retina, from Stargardt disease to recessive retinitis pigmentosa or cone-rod dystrophy [19,38,41]. A thorough discussion on the complex genetics of ABCR mutations and eye diseases is out of the scope of this article and can be found in [38]. No evidence on the molecular function of ABCR as a transporter has been provided, with the exception of the report that retinal activates its ATPase activity [42]. This makes retinal a good candidate as a substrate for ABCR-mediated transport, in analogy with the case of P-gp, whose enzymatic function can be stimulated by specific substrates [42–44].

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Cholesterol efflux regulatory protein, Tangier disease and familial high-density lipoprotein deficiency

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Cellular cholesterol efflux, by which cholesterol is transported from peripheral cells to HDL acceptor molecules for transport to the liver, is the first step of reverse cholesterol transport. Two genetic disorders, Tangier disease and some cases of familial HDL deficiency, have defects of cellular cholesterol efflux. The recent discovery of mutations in the *ABCI* gene, which encodes the cholesterol efflux regulatory protein, in both these disorders establishes cholesterol efflux regulatory protein as a rate-limiting factor in reverse cholesterol transport. *Curr Opin Lipidol* 11:117-122. © 2000 Lippincott Williams & Wilkins.

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Abbreviations

CERP	cholesterol efflux regulatory protein
FHA	familial HDL deficiency
MMP	matrix metalloproteinase
RCT	reverse cholesterol transport
SR-BI	scavenger receptor class B, type I

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Introduction

Intracellular cholesterol homeostasis in cells is exquisitely regulated and dependent on the balance between cholesterol synthesis and influx, its degradation, and cholesterol ester formation, translocation to the plasma membrane for efflux, and transport from the peripheral cell to the liver. Humans synthesize and degrade approximately 1 g cholesterol/day. About two-thirds of cholesterol is transported on LDL particles and approximately one-third of that cholesterol is deposited into extrahepatic tissues.

This concept of reverse cholesterol transport (RCT) was first proposed by Glomset in 1968 [1]. The efflux of cholesterol from cells represents the first step of RCT and can operate through a variety of mechanisms. Simple passive diffusion occurs when cholesterol is desorbed from the plasma membrane down the concentration gradient into the aqueous phase, and incorporated into phospholipid-containing acceptor particles. Active transport is a rate-limiting mechanism for removal of cholesterol from cells. In this process cellular cholesterol is transported to lipid depleted apolipoproteins [2]. In addition, scavenger receptor class B, type I (SR-BI)-mediated cholesterol exchange influences cholesterol flow into and out of specific cells. Interestingly, the mechanism by which SR-BI affects cholesterol efflux is not related to its ability to bind to a lipoprotein cholesterol acceptor. Cholesterol transport to phospholipid vesicles or cyclodextrins (neither of which binds to SR-BI) is also enhanced by SR-BI. There is some evidence that SR-BI alters the distribution of cholesterol in the cell, presumably making it more available for efflux (for review [3]). The rate of cholesterol efflux varies between cell types and is also influenced by the nature of the acceptor particle, with levels of HDL-phospholipids as major determinants of cholesterol efflux [4-9].

Recently, we and others [10**-12**] have identified the *ABCI* gene as a key determinant of active transport of cholesterol and phospholipids in certain cells. The clinical manifestations of Tangier disease with cholesterol ester accumulation in specific cell types, associated with premature coronary artery disease in some carriers for mutations, identifies the protein encoded by *ABCI*,

cholesterol efflux regulatory protein (CERP), as an important determinant of HDL levels in humans and an important component of the RCT pathway.

New insights concerning factors that influence cholesterol efflux from cells

Cells have evolved tightly regulated mechanisms for control of cholesterol concentrations. As cholesterol increases in cells, it may be stored in an acylated form produced by the action of acyl-coenzyme A-cholesterol-acyltransferases 1 and 2. Apolipoprotein AI can mobilize this stored cholesterol. Recent evidence has suggested that particular domains in apolipoprotein AI influence cholesterol efflux from cells. The carboxyl-terminal region of apolipoprotein AI encompassing residues 209–243 are important in promoting cholesterol efflux from cells [13,14], whereas apolipoprotein AI residues 143–164 appear to be crucial for lecithin:cholesterol acyltransferase activation [15–16].

For active transport of cholesterol from cells there is a need for appropriate ATP-dependent transfer of cholesterol and phospholipid, and also the requirement for an intact receptor molecule [17]. Further evidence for the importance of the carboxy terminus of apolipoprotein AI in mediating cholesterol efflux has been derived from studies of the influence of matrix metalloproteinases (MMPs), which reduce HDL-induced cholesterol efflux from human macrophage foam cells. The ability of several members of the MMP family to degrade human HDL and dramatically decrease cholesterol efflux highlights the importance of the integrity of the acceptors of cellular cholesterol and their role in cholesterol efflux [18]. This is consistent with studies [19] that show that trypsin treatment of HDL can abolish apolipoprotein-mediated cholesterol removal. The proteolytic modification of apolipoprotein AI by MMPs, and possibly by other as yet unknown molecules, may be crucial in blocking the physiological role of apolipoprotein AI in RCT from human atherosclerotic lesions. Further evidence of the importance of the availability of a lipid-poor apolipoprotein acceptor molecule comes from a recent study [20] that showed that the phospholipid transfer protein may aid in the generation of these acceptors and thereby enhance efflux.

It has previously been demonstrated that oxidative modification of LDL and its consequent accelerated uptake by arterial wall macrophages are important in the development of atherosclerosis. Similarly, HDL that contain high levels of polyunsaturated fatty acids (which can also be oxidized) are less effective in promoting cholesterol efflux from macrophage-derived foam cells. In contrast to oxidation, glycation of HDL appears to have no obvious effect on the capacity of HDL to mediate cholesterol efflux [21].

In addition to the extracellular cholesterol acceptor, cell type and intracellular environment may also influence cholesterol efflux [3**]. Metabolic manipulation of cells in culture may enhance apolipoprotein-mediated lipid release. Treatment of fibroblasts with interleukin-1 β has been shown [22] to increase efflux to apolipoprotein AI. An increase of intracellular free cholesterol increases both cholesterol and phospholipid efflux to apolipoproteins. Furthermore, cyclic AMP may also enhance specific binding of different apoproteins and transfer of lipids to the membrane. Cyclic AMP appears to impact on cholesterol efflux by increasing cholesterol ester hydrolysis, as well as the movement of free cholesterol from intracellular sites to the plasma membrane [23]. Interestingly, defects in this process have been reported in patients with Tangier disease [24]. In mouse macrophage cell lines, cyclic AMP causes a dramatic increase in internalization of apolipoprotein AI, followed by its resecretion in association with cholesterol, providing insights as to how apolipoprotein AI may stimulate efflux [25*].

Probucol has long been known to lower HDL-cholesterol levels, and one mechanism for this has now been shown to be reduced apolipoprotein AI-mediated cholesterol efflux. The effect of probucol on efflux appears to be generalized and not limited to specific cells. By contrast, a positive influence of cyclic AMP on cholesterol efflux appears to be cell-type specific and is only seen, for example, in certain macrophage cell lines, and not in human fibroblasts. This also suggests that some, but not all, influences on cholesterol efflux are cell-specific [23].

SR-BI is a scavenger receptor that binds HDL with high affinity and mediates selective uptake of cholesteryl ester from HDL. Recently, it has also been demonstrated that SR-BI can stimulate free cholesterol flux from Chinese hamster ovary cells that express mouse SR-BI, demonstrating that SR-BI can mediate alterations to membrane cholesterol traffic, provoking bidirectional cholesterol flux [26,27].

Cholesterol efflux regulatory protein, Tangier disease, and HDL deficiency

In humans, defects in cholesterol efflux are most evident in patients with Tangier disease, which manifests with marked deficiency of HDL and cholesterol ester accumulation in specific cells. Furthermore, depressed levels of cholesterol efflux are also seen in some patients with familial HDL deficiency (FHA). Recently, mutations in the *ABCI* gene were found to underlie both Tangier disease and this form of FHA.

The gene for Tangier disease was mapped to 9q31 towards the end of 1998 [28]. Thereafter, different approaches were taken in different laboratories to clone

the Tangier disease gene. These included analysis of expression of genes moderated by cholesterol accumulation in cells [11**,12**], followed by mapping these to specific regions of the human genome, as well as by classical positional cloning strategies [10**].

We undertook two separate classical positional cloning approaches to identify the genes that underlie Tangier disease and FHA [10**]. To that point, it had been recognized that FHA had features of an autosomal dominant disorder characterized by clear manifestations of low HDL in the heterozygote, with no obvious features of cholesterol ester deposition in cells [29]. By contrast, Tangier disease had been defined as an autosomal recessive illness with clear evidence for cholesterol deposition in cells and less obvious manifestations in the heterozygote. Furthermore, even though both diseases had some evidence for cholesterol efflux, this did not necessarily mean that this was due to defects in the same gene. Numerous genetic disorders with common phenotypic features may be caused by defects in different genes (e.g. genes affecting hypertension). In addition, in different diseases, defects in multiple genes may be associated with a very similar phenotype (for example diabetes and Hirschsprung's disease).

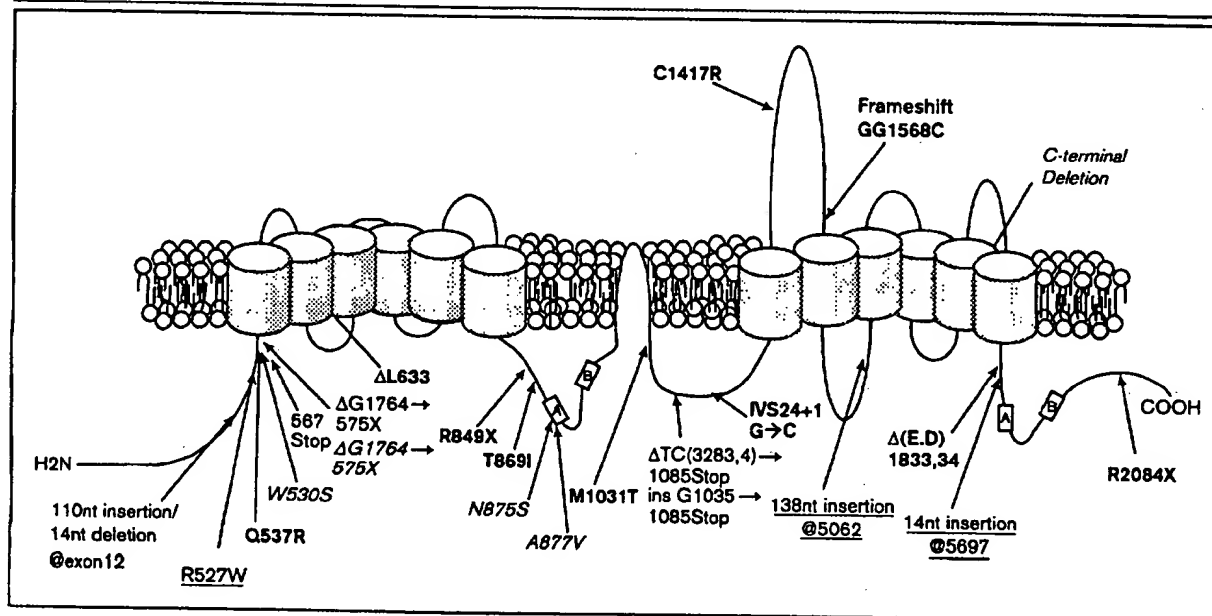
Genetic analysis revealed that the gene for FHAI mapped to the same region as the mutation that

underlies Tangier disease [10**]. This suggested that Tangier disease and FHA were due to mutations in the same gene, or potentially these mutations were due to different genes in close proximity to each other. Further analysis, however, revealed that these diseases were allelic with Tangier disease, resulting from mutations on both alleles of *ABCI*, whereas FHAI was due to a mutation on a single allele.

ABCI is a member of the ATP-binding cassette family of genes, a large and rapidly expanding gene family currently containing about 50 members. Although all *ABC* family members contain an ATP-binding cassette that consists of consensus Walker A and Walker B motifs, as well as membrane-spanning sequences, they vary widely in the arrangement of their protein domains. Some, referred to as 'half-transporters', contain only one ATP-binding site and a transmembrane domain consisting of six membrane-spanning segments. *ABCI* itself contains two ATP-binding cassettes, two membrane-spanning domains with six transmembrane domains each, a large extracellular loop and a long amino-terminal segment (Fig. 1).

Heterozygotes for Tangier disease are genetically similar to those of patients with FHA. Why, then, was Tangier disease regarded as inherited as a recessive trait, whereas FHA was presumed to be dominant? Analysis of the

Figure 1. Schematic diagram of the cholesterol efflux regulatory protein



Schematic diagram of the cholesterol efflux regulatory protein, showing the location of all mutations reported to date ([12**] - italics; [11**] - light grey; [10**,30**] - black; [31**] - underlined). The critical ATP-binding cassette region is characterized by the Walker A and B motifs, designated A and B, respectively.

families [10**,30*] provides some explanation. Dominance implies that the phenotype is manifest in the heterozygote. Our first families assessed for FHA in general had more severe mutations that were associated more frequently with HDL levels below 5% for age and sex (Fig. 1). These low HDL levels increased the likelihood of ascertainment. Mutations that result in truncation of the protein or major deletions are more often seen in persons with HDL below 5%. By contrast, the two families with Tangier disease that we studied [10**] had missense mutations and, in all likelihood, these mutations may give rise to proteins with significant functional deficits, but retaining some residual activity (Fig. 2).

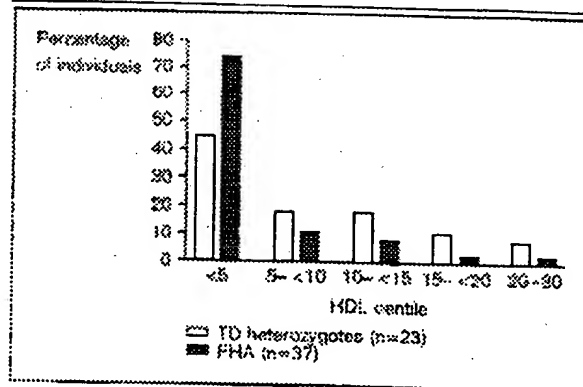
The *ABCR* gene, which encodes a retinal rod photoreceptor protein, has the strongest homology to CERP (approximately 50%) of any ABC protein. Interestingly, persons who are heterozygous for mutations in *ABCR* have a phenotype (e.g. adult-onset muscular dystrophy), which was not previously thought to be related to the phenotype of retinitis pigmentosa that is due to mutations on both alleles [32]. For mutations in *ABCR* and *ABCI*, the clinical spectrum is likely to vary dependent on the functional effects on the protein. Thus, we would predict a broader clinical spectrum for patients with mutations on both alleles, particularly when one of these alleles results in an *ABCI* protein with some residual activity.

The distribution of Tangier disease and FHA mutations in the *ABCI* gene can provide information about the role of CERP and its different domains. Many of the missense mutations seen in Tangier disease or FHA families occur in or near the ATP-binding cassettes, with fewer mutations in the membrane-spanning segments (Fig. 1). This is consistent with active cholesterol efflux being an energy-requiring process that is dependent on ATP hydrolysis.

Another important question is whether different sites of mutation are more likely to be associated with a particular phenotype, in particular coronary artery disease. Preliminary assessment has revealed that the likelihood of coronary disease does not correlate with the location of the mutation. Rather, it is likely to be reflective of the remaining function of CERP, in addition to other genetic and environmental factors.

Mutations in CERP are associated with significant defects in cholesterol efflux. The fact that patients with Tangier disease have such marked reduction in efflux demonstrates the importance of CERP *in vivo* in the efflux of cholesterol or phospholipid from cells, and the importance of this first step in the regulation

Figure 2. HDL-cholesterol levels



HDL-cholesterol levels in heterozygous carriers of cholesterol efflux regulatory protein (CERP) mutations from Tangier disease (TD) and familial HDL deficiency (FHA) families are shown. The percentage of individuals from TD or FHA families with HDL-cholesterol levels falling in a given range of centiles for age and sex are plotted. Individuals from the FHA families (n=37) are more often found with HDL in the lower centile categories than those from the TD families (n=23).

of HDL levels in humans. Clearly, lipidation of nascent HDL represents a critical step in the maintenance and regulation of HDL levels in humans (Fig. 3).

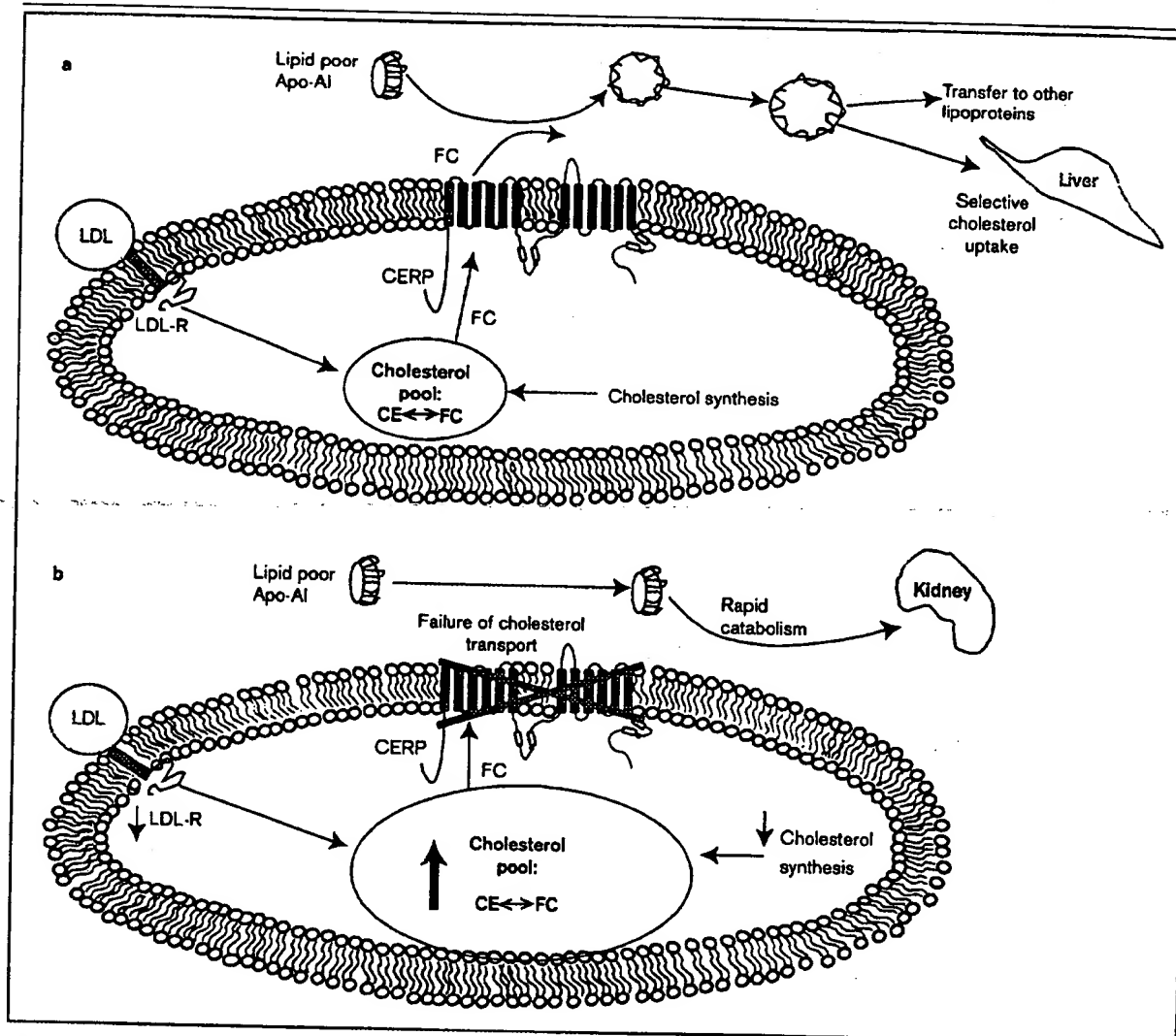
An important question is whether defects in other proteins are likely to be contributing to this major deficit in efflux. Clearly, mutations in other proteins can be associated with defects in cholesterol efflux, such as mutations in particular domains of apolipoprotein AI. Analysis of the majority of the patients and families who presented with cholesterol efflux defects, however, has revealed that mutations in CERP represent the major but not the only determinant for cholesterol efflux in humans.

Determination of HDL levels is multigenic and it is not surprising that the phenotype in heterozygotes for CERP deficiency varies not only on the basis of the nature of the mutation, but also on the basis of other factors that may influence HDL levels. For example, age appears to partly influence the manifestation of the phenotype, with more variability of the phenotype seen in patients less than 20 years of age (data not shown).

Conclusion

Discovery of the effect of CERP in the determination of HDL levels in humans confirms the role of RCT in the maintenance of HDL levels in humans. Although CERP is a major determinant of efflux, clearly other as yet

Figure 3. HDL metabolism



HDL metabolism in the presence (a) and absence (b) of cholesterol efflux regulatory protein (CERP) function. When CERP is present and cholesterol is efficiently mobilized from intracellular stores and transferred to lipid-poor apolipoprotein AI (Apo-AI), forming mature HDL particles, which can then function in the reverse cholesterol transport process. In the absence of CERP function, cholesterol ester accumulates within the cell, and the lipid-poor Apo-AI particles are not lipidated and are rapidly catabolized. CE, cholesterol ester; FC, free cholesterol; LDL-R, LDL receptor.

undiscovered proteins are likely to influence this process. Identification of CERP has provided a critical platform from which we may now begin to elucidate the functioning of the RCT pathway, and the regulation of HDL-cholesterol levels in humans.

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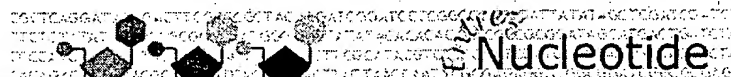
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Nucleotide

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Books

Search Nucleotide for Go Clear

Limits Preview/Index History Clipboard Details

Display default Show: 20 Send to File Get Subsequence

☐ 1: AJ012376. Homo sapiens mRNA...[gi:4128032][Links](#)

LOCUS HSA012376 6880 bp mRNA linear PRI 13-NOV-2001
DEFINITION Homo sapiens mRNA for ATP-binding cassette transporter-1 (ABC-1).
ACCESSION AJ012376
VERSION AJ012376.1 GI:4128032
KEYWORDS ABC-1 gene; ATP-binding cassette transporter-1.
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1
AUTHORS Langmann,T., Klucken,J., Reil,M., Liebisch,G., Luciani,M.F.,
Chimini,G., Kaminski,W.E. and Schmitz,G.
TITLE Molecular cloning of the human ATP-binding cassette transporter 1
(hABC1): evidence for sterol-dependent regulation in macrophages
JOURNAL Biochem. Biophys. Res. Commun. 257 (1), 29-33 (1999)
MEDLINE 99194549
PUBMED 10092505
REFERENCE 2
AUTHORS Langmann,T., Klucken,J., Reil,M., Liebisch,G., Luciani,M.F.,
Chimini,G., Kaminski,W. and Schmitz,G.
TITLE Molecular Cloning of the Human ATP-Binding Cassette Transporter 1
(hABC1): Evidence for Dterol-Dependent Regulation in Macrophages
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 97, 7987-7992 (2000)
REFERENCE 3 (bases 1 to 6880)
AUTHORS Langmann,T.
TITLE Direct Submission
JOURNAL Submitted (11-NOV-1998) Langmann T., Institute for Clinical
Chemistry and Laboratory Medicine, University of Regensburg,
Franz-Josef-Strauss-Allee 11, 93053, GERMANY
FEATURES
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6727..6880

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3'UTR

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301	agcatg	aagg acatgc	caa agttct	gaga acattac	agc agatca	agaa atccag	ctca
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1081	gggaag	atcc tgtata	cacc tgacac	tcca gccaca	aggg aggtc	atggc tga	gtg
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1321	caagac	atcg tggcg	ttttt ggcca	agcac ccagag	gatg tccag	tccag taatg	gttct
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1561	attact	ccag gcagc	attga gctg	cccat catgt	caagt acaag	atccg aatg	gacatt

This is not Langmann's sequence (ie this is not Swiss protein: 095477)

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			Indels	0
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DB 61	MSAGTLPWQGIICNANPCFRPTGEPAGVGVGNFNKSIARLFSADARRLLLSQDIT	120		
QY 121	SHKMRKYLRTLQIKKSSNLKQDFLVDNETFSGFLYBNLSLPSKSTYDKMLRADVILH	180		
DB 121	SHKMRKYLRTLQIKKSSNLKQDFLVDNETFSGFLYBNLSLPSKSTYDKMLRADVILH	180		
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QY 961	GKDIRSEMSTIRQNLGVCPQHNVLFDMLVIEHIMFYARLKLGLSKHYKAEQOMALDVG	1020		
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QY 1681	VSKAKHLOFISGVKPVITWLSNFVMDNCYVVPATVITVIFCFOOKSVYSTNLPLVAL	1740
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DB 1741	LLLLYGNSTPLMTPASFTVKIPSTAYVLTSVNLFIGINGSVATFVLELFTDNKLNNIN	1800
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DB 1801	DILKSVFLIFPHFCLGRLIDWKNQAMADALERFQENRFPVPSLWDLVGNLFAVVEG	1860
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QY 1921	YRKRKRPVADRICVIGIPGECFGLLVNGAGKSSFTPKMLTGTDTTTRGDALFNKNSILSN	1980
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QY 2101	SHSBECEALCTRNAIKVNGFRCLSGVQHLKRNFGDGYTIVVIRIAGSNPLKPVQDFFG	2160

RESULT 2					
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AC	C41233;				
AD	01-FEB-1995 (Rel. 31, Created)				
AT	16-OCT-2001 (Rel. 40, Last sequence update)				
DT	15-JUN-2002 (Rel. 41, Last annotation update)				
DE	ATP-binding cassette, sub-family A, member 1 (ATP-binding cassette transporter 1) (ATP-binding cassette 1) (ABC-1).				
DG	ABCA1 OR ABCI1.				
DN	Mus musculus (Mouse).				
OS	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;				
OC	Mammalia; Mitharia; Rodentia; Sciurognathi; Muridae; Murinae; Mus.				
OX	NCBI_TaxId=10090;				
ON	[1]				
RP	SEQUENCE FROM N.A.				
RC	STRAIN=DNA/2; TISSUE=Macrophage;				
RX	MEDLINE=94375008; PubMed=8088782;				
RA	Luciani M.F., Deaknot F., Savary S., Mattei M.-G., Chimini G.;				
RT	"Cloning of two novel ABC transporters mapping on human chromosome 9.";				
RL	Genomics 21:150-159(1994).				
RN	[2]				
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CP	STRAIN=C57BL/6J;				
RC	Qiu Y., Cavellier L., Chiu S., Rubin E., Cheong J.-F.;				
RA	"Human and mouse ABCA1 comparative sequencing and transgenesis studies identify potential regulatory sequences.";				
RT	Submitted (JUL-2000) to the ENML/genBank/DBJ databases.				
CC	-!- FUNCTION: CAMP-DEPENDENT AND SULFOFLUOREA-SENSITIVE ANION TRANSPORTER. KEY GATEKEEPER INFLUENCING INTRACELLULAR CHOLESTEROL TRANSPORT (BY SIMILARITY).				
CC	-!- TISSUE SPECIFICITY: WIDELY EXPRESSED IN ADULT TISSUES. HIGHEST LEVELS ARE FOUND IN PREGNANT UTERUS AND UTERUS.				
CC	-!- DOMAIN: MULTIFUNCTIONAL POLYPEPTIDE WITH TWO HOMOLOGOUS HALVES, EACH CONTAINING AN HYDROPHOBIC MEMBRANE-ANCHORING DOMAIN AND AN ATP BINDING CASSETTE (ABC) DOMAIN.				
CC	-!- SIMILARITY: BELONGS TO THE ABC TRANSPORTER FAMILY. ABCA SUBFAMILY.				
CC	This SWISS-PROT entry is copyright. It is produced through a collaboration between the Swiss Institute of Bioinformatics and the EMBL outstation at the European Bioinformatics Institute. There are no restrictions on use by non-profit institutions as long as its content is in no way modified and this statement is not removed. Usage by and for commercial entities requires a license agreement (See http://www.isb-sib.ch/announcements or send an email to license@isb-sib.ch).				
DR	EMBL; X75926; CAA53530.1; AUT_INIT.				
DR	EMBL; AF287263; AAC39073.1; ALT_INIT.				
DR	MGI; MG1:99607; Abca1.				
DR	InterPro; IPR003593; AAA_ATPase.				
DR	InterPro; IPR003439; ABC_Transportr.				
DR	Pfam; PF00005; ABC_tran; 2.				
DR	ProDom; PD000006; ABC_Transportrr; 2				
DR					
DR					
KW	ATP-binding; Glycoprotein; Transmembrane; Transport.				
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FT	TRANSMEN 640 556 POTENTIAL.				
FT	TRANSMEN 690 706 POTENTIAL.				
FT	TRANSMEN 717 733 POTENTIAL.				

Query Match	95.4%;	Score 11256;	DB 1;	Length
Best Local Similarity	95.0%;	Prod. No. 0;		
Matches 2149;	Conservative	55;	Mismatches	57;

[illegible]

UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 99,395-A)

IN THE APPLICATION OF:

Lawn et al.

Serial No. 09/595,526

Filed: June 16, 2000

Title: Compositions and Methods
For Increasing Cholesterol
Efflux and Raising HDL,
Using ATP Binding Cassette
Transporter Protein ABC1

Examiner: Rao, Manjunath N.

Group Art Unit: 1652

Confirmation No.: 9969

DECLARATION OF RICHARD LAWN, PH.D. UNDER 37 C.F.R. §1.131Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Richard Lawn, Ph.D., declare as follows:

- (1) I am a named inventor of the above-mentioned U.S. Patent Application Serial Number 09/595,526.
- (2) The inventions described in the claims of Patent Application Serial Number 09/595,526 were conceived and reduced to practice cooperatively by the named inventors, Dr. Richard Lawn, Dr. David Wade, Dr. Michael Garvin, and Dr. John Oran, prior to May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.
- (3) The amino acid sequence of ABC1 protein, which appears as SEQ ID NO: 2 in the above-mentioned application, and the corresponding nucleotide sequence of the open reading frame of the ABC1 gene (cDNA), which appears as nucleotides 291-7074 of SEQ ID NO: 1 in the above-mentioned

application, were reduced to practice before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

- (4) The nucleotide sequence of ABC1 gene (cDNA) including the 5' and 3' untranslated sequences, which appears as SEQ ID NO: 1 in the above-mentioned application, was reduced to practice before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.
- (5) Attached as Exhibit A is a copy of two laboratory notebook pages (from which the dates have been redacted) that demonstrate that the cDNA clone of the ABC1 gene was isolated before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.
- (6) Attached as Exhibit B is a copy of the 4-page sequence report (from which the dates have been redacted) that demonstrates that the nucleotide sequence of ABC1 gene (cDNA), which appears as SEQ ID NO: 1 in the above-mentioned application was sequenced before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.
- (7) Attached as Exhibit C is a copy of four laboratory notebook pages (from which the dates have been redacted) that demonstrate that the extended open reading frame of ABC-1 gene (cDNA; nucleotides 291-7074 of SEQ ID NO: 1) was isolated before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.
- (8) Attached as Exhibit D is a copy of a one-page sequence report (from which the dates have been redacted) that demonstrates that the amino acid sequence of ABC1 protein (predicted from translation of the nucleotide sequence) which appears as SEQ ID NO: 2 in the above-mentioned application was sequenced before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.
- (9) I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge

imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Respectfully submitted,

Date: Sept 25, 2003

By: Richard Lawn
Richard Lawn, Ph.D.

TITLE _____

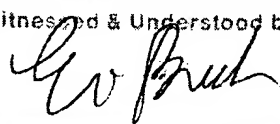
From Page No. _____

Selecting ABC1 from pCEP4 library using RecA kit

1. Isolate total library DNA using Qiagen kit.
2. ~~Label~~ Label 600 bp PCR product from 5' end of ABC1 using biotin.
3. Select according to Clontech instructions.
4. Transform 1 μ l of plasmid DNA into DH10B electrocompetent cells.
Got ~ 7000 colonies total.
5. Isolate plasmid from all the transformants after O.N. growth in amp.
6. Carry another round of ~~selecting~~ selection under the same conditions as round one, and transform DH10B w/ 1 μ l of DNA.
Got ~ 150,000 colonies total.
7. Spread 1000 cells on one 150 mm agar plate from round one,
and 500 cells " " " " two.
8. Do colony lift on the plates and hybridize to the 600 bp probe.
found 0 positives on round 1 filter, 3 positives on round 2 filter.
the 3rd colony was too close to another colony, so picked the 4th colony in case of misalignment.

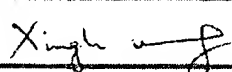
To Page No. _____

Witnessed & Understood by me,



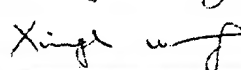
Date ,

Invented by



Date

Recorded by



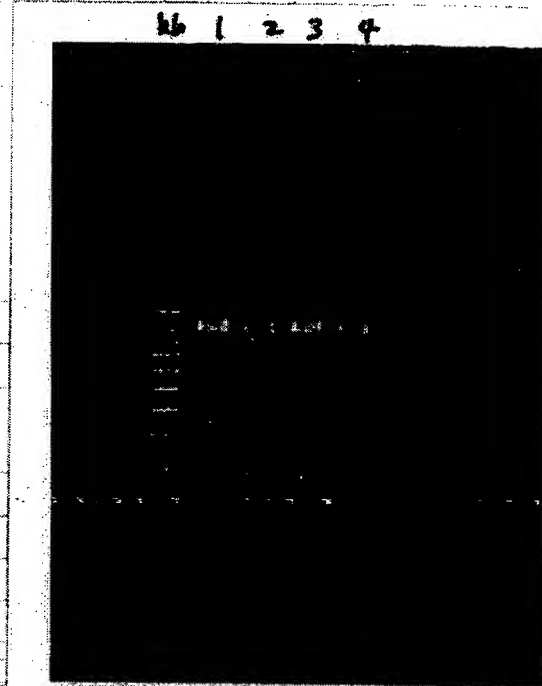
TITLE _____

From Page No. _____

9. prepare miniprep plasmid DNA and cut w/
Hind III & Xho I (cloning sites)

colony 1 showed two fragments of
5.5 kb & 2.9 kb

colony 2 & 3 showed very weak ~1 kb
bands, but not captured by the
photograph



10. PCR to confirm the existence of the
probe sequence in the picked clones.

Using the primers at the ends of the
600 bp probe.

Template:

Lane P. 10 ng of probe

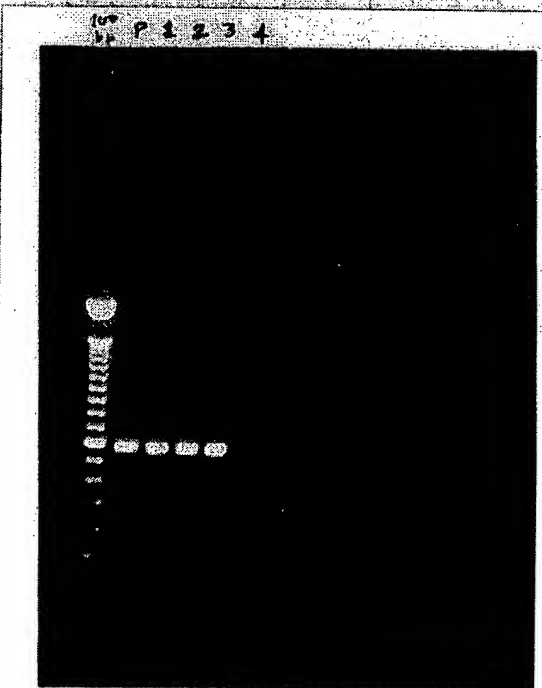
1. 1 µl of clone 1 miniprep

2. " 2

3. " 3

4. " 4

clone 4 showed no band at all, indicating
the absence of PCR contamination.



To Page No. _____

Witnessed & Understood by me,

Eric Burk

Date /

Invented by

Xing w f

Date

Recorded by

Xing w f

CVTHABC1 NUCLEOTIDE 10442 BP

Start codon: 291

Stop codon :7074

Genbank CDS : 350 to 7230

Overlap with genomic fragment pAPR1 5' promoter: 1 to 67

```
1  GGCCGGGACC CGCAGAGCCG AGCCGACCTT TCTCTCCCGG GCTGCGGCAG GGCAGGGCGG
61  GGAGCTCCGC GCACCAACAG AGCCGGTTCT CAGGGCGCTT TGCTCCTTGT TTTTCCCGG
121 GTTCTGTTTT CTCCCCTTCT CCGGAAGGCT TGTCAAGGGG TAGGAGAAAG AGACGCAAAC
181 ACAAAAGTGG AAAACAGTTA ATGACCAGCC ACGGGCGTCC CTGCTGTGAG CTCTGGCCGC
241 TGCCTTCCAG GGCTCCCGAG CCACACGCTG GCGTGCTGG CTGAGGGAAC ATGGCTTGTT
301 GGCCTCAGCT GAGGTTGCTG CTGTGGAAGA ACCTCACTTT CAGAAGAAGA CAAACATGTC
361 AGCTGTTACT GGAAGTGGCC TGGCCTCTAT TTATCTTCCT GATCCTGATC TCTGTTCCGC
421 TGAGCTACCC ACCCTATGAA CAACATGAAT GCCATTTTCC AAATAAAGCC ATGCCCTCTG
481 CAGGAACACT TCCTTGGGTT CAGGGGATTA TCTGTAATGC CAACAACCCC TGTTTCCGTT
541 ACCCGACTCC TGGGGAGGCT CCCGGAGTTG TTGGAACCTT TAACAAATCC ATTGTGGCTC
601 GCCTGTTCTC AGATGCTCGG AGGCTTCTTT TATACAGCCA GAAAGACACC AGCATGAAGG
661 ACATCGCAGG AGTTCTGAGA ACATTACAGC AGATCAAGAA ATCCAGCTCA AACTTGAAGC
721 TTCAAGATTT CCTGGTGGAC AATGAAACCT TCTCTGGGTT CCTATATCAC AACCTCTCTC
781 TCCCAAAGTC TACTGTGGAC AAGATGCTGA GGGCTGATGT CATTCTCCAC AAGGTATTTT
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901 TTCAACTTGG TGACCAAGAA GTTTCTGAGC TTTGTGGCCT ACCAAAGGAG AAAGTGGCTG
961 CAGCAGAGCG AGTACTTCGT TCCAACATGG ACATCCTGAA GCCAATCCTG AGAACAATAA
1021 ACTCTACATC TCCCTTCCCG AGCAAGGAGC TGGCTGAAGC CACAAAAACA TTGCTGCATA
1081 GTCTTGGGAC TCTGGCCCAG GAGCTGTTCA GCATGAGAAG CTGGAGTGAC ATGCGACAGG
1141 AGGTGATGTT TCTGACCAAT GTGAACAGCT CCAGCTCCTC CACCCAAATC TACCAGGCTG
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1381 CTAGTCTCTT TTCCCGCATT ATCTGGAAAG CTCTGAAGCC GCTGCTCGTT GGAAGATCC
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1501 AGGAACTGGC TGTGTTCCAT GATCTGGAAG GCATGTGGGA GGAATCAGC CCCAAGATCT
1561 GGACCTTCAT GGAGAACAGC CAAGAAATGG ACCTTGTCCG GATGCTGTTG GACAGCAGGG
1621 ACAATGACCA CTTTGGGAA CAGCAGTTGG ATGGCTTAGA TTGGACAGCC CAAGACATCG
1681 TGGCGTTTTT GGCCAAGCAC CCAGAGGATG TCCAGTCCAG TAATGGTTCT GTGTACACCT
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1801 GTGTCAACCT GAACAAGCTA GAACCCATAG CAACAGAAGT CTGGCTCATC AACAAAGTCCA
1861 TGGAGCTGCT GGATGAGAGG AAGTTCTGGG CTGGTATTGT GTTCACTGGA ATTACTCCAG
1921 GCAGCATTGA GCTGCCCCAT CATGTCAAGT ACAAGATCCG AATGGACATT GACAATGTGG
1981 AGAGGACAAA TAAAATCAAG GATGGGTACT GGGACCCTGG TCCTCGAGCT GACCCCTTTG
2041 AGGACATGCG GTACGTCTGG GGGGGCTTCG CCTACTTGCA GGATGTGGTG GAGCAGGCAA
2101 TCATCAGGGT GCTGACGGGC ACCGAGAAGA AAAGTGGTGT CTATATGCAA CAGATGCCCT
2161 ATCCCTGTTA CGTTGATGAC ATCTTTCTGC GGGTGATGAG CCGGTCAATG CCCCTCTTCA
2221 TGACGCTGGC CTGGATTTAC TCAGTGGCTG TGATCATCAA GGGCATCGTG TATGAGAAGG
2281 AGGCACGGCT GAAAGAGACC ATGCGGATCA TGGGCCTGGA CAACAGCATA CTCTGGTTTA
2341 GCTGGTTCAT TAGTAGCCTC ATTCTCTTTC TTGTGAGCGC TGGCCTGCTA GTGGTCATCC
2401 TGAAGTTAGG AAACCTGCTG CCCTACAGTG ATCCCAGCGT GGTGTTTGTC TTCCTGTCCG
2461 TGTTTGCTGT GGTGACAATC CTGCAGTGCT TCCTGATTAG CACACTCTTC TCCAGAGCCA
2521 ACCTGGCAGT AGCCTGTGGG GGCATCATGT ACTTCACGCT GTACCTGCCC TACGTCCTGT
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2701 TGCAGTGGGA CAACCTGTTT GAGAGTCCTG TGGAGGAAGA TGGCTTCAAT CTCACCACTT
2761 CGATCTCCAT GATGCTGTTT GACACCTTCC TCTATGGGGT GATGACCTGG TACATTGAGG
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2941 CAGAAATCTG CATGGAGGAG GAACCCACCC ACTTGAAGCT GGGCGTGTCC ATTCAGAACC
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3181	TTCGCTCTGA	GATGAGCACC	ATCCGGCAGA	ACCTGGGGGT	CTGTCCCCAG	CATAACGTGC
3241	TGTTTGACAT	GCTGACTGTC	GAAGAACACA	TCTGGTTCTA	TGCCCCGCTT	AAAGGGCTCT
3301	CTGAGAAGCA	CGTGAAGGCG	GAGATGGAGC	AGATGGCCCT	GGATGTTGGT	TTGCCATCAA
3361	GCAAGCTGAA	AAGCAAAACA	AGCCAGCTGT	CAGGTGGAAT	GCAGAGAAAG	CTATCTGTGG
3421	CCTTGCCCTT	TGTCGGGGGA	TCTAAGGTTG	TCATTCTGGA	TGAACCCACA	GCTGGTGTGG
3481	ACCCTTACTC	CCGCAGGGGA	ATATGGGAGC	TGCTGCTGAA	ATACCGACAA	GGCCGCACCA
3541	TTATTCTCTC	TACACACCAC	ATGGATGAAG	CGGACGTCCT	GGGGGACAGG	ATTGCCATCA
3601	TCTCCCATGG	GAAGCTGTGC	TGTGTGGGCT	CCTCCCTGTT	TCTGAAGAAC	CAGCTGGGAA
3661	CAGGCTACTA	CCTGACCTTG	GTCAAGAAAG	ATGTGGAATC	CTCCCTCAGT	TCCTGCAGAA
3721	ACAGTAGTAG	CACTGTGTCA	TACCTGAAAA	AGGAGGACAG	TGTTTCTCAG	AGCAGTTCTG
3781	ATGCTGGCCT	GGGCAGCGAC	CATGAGAGTG	ACACGCTGAC	CATCGATGTC	TCTGCTATCT
3841	CCAACCTCAT	CAGGAAGCAT	GTGTCTGAAG	CCCGGCTGGT	GGAAGACATA	GGGCATGAGC
3901	TGACCTATGT	GCTGCCATAT	GAAGCTGCTA	AGGAGGGAGC	CTTTGTGGAA	CTCTTTTCATG
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4141	CGTTCACTGA	AGATGATGCT	GCTGATCCAA	ATGATTCTGA	CATAGACCCA	GGCTCCAGAG
4201	AGACAGACTT	GCTCAGTGGG	ATGGATGGCA	AAGGGTCCTA	CCAGGTGAAA	GGCTGGAAAC
4261	TTACACAGCA	ACAGTTTGTG	GCCCTTTTGT	GGAAGAGACT	GCTAATTGCC	AGACGGAGTC
4321	GGAAAGGATT	TTTTGCTCAG	ATTGTCTTGC	CAGCTGTGTT	TGTCTGCATT	GCCCTTGTGT
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5221	CAGAGGTGGC	TCTGATGACC	ACATCAGTGG	ATGTCCTTGT	GTCCATCTGT	GTCATCTTTG
5281	CAATGTCCTT	CGTCCCAGCC	AGCTTTGTCT	TATTCCTGAT	CCAGGAGCGG	GTCAGCAAAG
5341	CAAAACACCT	GCAGTTCATC	AGTGGAGTGA	AGCCTGTCAT	CTACTGGCTC	TCTAATTTTG
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5461	TCCAGCAGAA	GTCCTATGTG	TCCTCCACCA	ATCTGCCTGT	GCTAGCCCTT	CTACTTTTGC
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5701	AGTCCGTGTT	CTTGATCTTC	CCACATTTTT	GCCTGGGACG	AGGGCTCATC	GACATGGTGA
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5941	AGCTATCTCC	TCTGAATGAT	GAAGATGAAG	ATGTGAGGCG	GGAAAGACAG	AGAATTCCTG
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6121	TCCTGGGAGT	TAATGGGGCT	GGAAAATCAT	CAACTTTCAA	GATGTAAACA	GGAGATACCA
6181	CTGTTACCAG	AGGAGATGCT	TTCCTTAACA	AAAATAGTAT	CTTATCAAAC	ATCCATGAAG
6241	TACATCAGAA	CATGGGCTAC	TGCCCTCAGT	TTGATGCCAT	CACAGAGCTG	TTGACTGGGA
6301	GAGAACACGT	GGAGTTCTTT	GCCCTTTTGA	GAGGAGTCCC	AGAGAAAGAA	GTTGGCAAGG
6361	TTGGTGAGTG	GGCGATTCCG	AAACTGGGCC	TCGTGAAGTA	TGGAGAAAAA	TATGCTGGTA
6421	ACTATAGTGG	AGGCAACAAA	CGCAAGCTCT	CTACAGCCAT	GGCTTTGATC	GGCGGGCCTC

6481	CTGTGGTGT	TCTGGATGAA	CCCACCACAG	GCATGGATCC	CAAAGCCCGG	CGGTTCTTGT
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6661	GCCTTGGCAG	TGTCCAGCAT	CTAAAAAATA	GGTTTGGAGA	TGGTTATACA	ATAGTTGTAC
6721	GAATAGCAGG	GTCCAACCCG	GACCTGAAGC	CTGTCCAGGA	TTTCTTTGGA	CTTGCAATTC
6781	CTGGAAGTGT	TCTAAAAGAG	AAACACCGGA	ACATGCTACA	ATACCAGCTT	CCATCTTCAT
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6961	AAAGTGATGA	TGACCACTTA	AAAGACCTCT	CATTACACAA	AAACCAGACA	GTAGTGGACG
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7321	ACCTATACCT	ATGTGAAACT	CTATTATGGA	ACCCAATGGA	CATATGGGTT	TGAACACACA
7381	CTTTTTTTTT	TTTTTTGTTC	CTGTGTATTC	TCATTGGGGT	TGCAACAATA	ATTTCATCAAG
7441	TAATCATGGC	CAGCGATTAT	TGATCAAAAT	CAAAAGGTAA	TGCACATCCT	CATTCATAA
7501	GCCATGCCAT	GCCCAGGAGA	CTGGTTTCCC	GGTGACACAT	CCATTGCTGG	CAATGAGTGT
7561	GCCAGAGTTA	TTAGTGCCAA	GTTTTTCAGA	AAGTTTGAAG	CACCATGGTG	TGTCATGCTC
7621	ACTTTTGTGA	AAGCTGCTCT	GCTCAGAGTC	TATCAACATT	GAATATCAGT	TGACAGAATG
7681	GTGCCATGCG	TGGCTAACAT	CCTGCTTTGA	TTCCCTCTGA	TAAGCTGTTT	TGGTGGCAGT
7741	AACATGCAAC	AAAAATGTGG	GTGTCTCTAG	GCACGGGAAA	CTTGGTTCCA	TTGTTATATT
7801	GTCCTATGCT	TCGAGCCATG	GGTCTACAGG	GTCATCCTTA	TGAGACTCTT	AAATATACTT
7861	AGATCCTGGT	AAGAGGCAAA	GAATCAACAG	CCAAACTGCT	GGGGCTGCAA	GCTGCTGAAG
7921	CCAGGGCATG	GGATTAAAGA	GATTGTGCGT	TCAAACCTAG	GGAAGCCTGT	GCCCATTTGT
7981	CCTGACTGTC	TGCTAACATG	GTACACTGCA	TCTCAAGATG	TTTATCTGAC	ACAAGTGTAT
8041	TATTTCTGGC	TTTTTGAATT	AATCTAGAAA	ATGAAAAGAT	GGAGTTGTAT	TTTGACAAAA
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8401	CGTATAAGAC	TAGAGAGATA	TTAAGTCTCA	GTACACTTCC	TGTGCCATGT	TATTCAGCTC
8461	ACTGGTTTAC	AAATATAGGT	TGCTTTGTGG	TTGTAGGAGC	CCACTGTAAC	AAATATGGGC
8521	AGCCTTTTTT	TTTTTTTTTT	AATTGCAACA	ATGCAAAAGC	CAAGAAAGTA	TAAGGGTCAC
8581	AAGTTTAAAC	AATGAATTCT	TCAACAGGGA	AAACAGCTAG	CTTGAAAAC	TGCTGAAAAA
8641	CACAACTTGT	GTTTATGGCA	TTTAGTACCT	TCAAATAATT	GGCTTTGCAG	ATATTGGATA
8701	CCCCATTAAA	TCTGACAGTC	TCAAATTTTT	CATCTCTTCA	ATCACTAGTC	AAGAAAAATA
8761	TAAAAACAAC	AAATACTTCC	ATATGGAGCA	TTTTTCAGAG	TTTTCTAACC	CAGTCTTATT
8821	TTTCTAGTCA	GTAACATTTT	GTAATAATAC	TGTTTCACTA	ATACTTACTG	TTAACTGTCT
8881	TGAGAGAAAA	GAAAAATATG	AGAGAACTAT	TGTTTGGGGA	AGTTCAAGTG	ATCTTTCAAT
8941	ATCATTACTA	ACTTCTTCCA	CTTTTTCCAA	AATTTGAATA	TTAACGCTAA	AGGTGTAAGA
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9061	CTGCTGAAAA	AGAAAAAAAT	GATTGTTTTA	GAAGTTAAAG	TCAATATTGA	TTTTAAATAT
9121	AAGTAATGAA	GGCATATTTT	CAATAACTAG	TGATATGGCA	TCGTTGCAAT	TTACAGTATC
9181	TTCAAAAATA	CAGAATTTAT	AGAATAATTT	CTCCTCATT	AATATTTTTT	AAAATCAAGG
9241	TTATGGTTTC	CTCATTTTAC	TAAAATCGTA	TTCTAATTCT	TCATTATAGT	AAATCTATGA
9301	GCAACTCCTT	ACTTCGGTTC	CTCTGATTTT	AAGGCCATAT	TTTAAAAAAT	CAAAAGGCAC
9361	TGTGAACTAT	TTTGAAGAAA	ACACGACATT	TTAATACAGA	TTGAAAGGAC	CTCTTCTGAA
9421	GCTAGAAACA	ATCTATAGTT	ATACATCTTC	ATTAATACTG	TGTTACCTTT	TAAAAATAGTA
9481	ATTTTTTACA	TTTTCTCTGT	TAAACCTAAT	TGTGGTAGAA	ATTTTTACCA	ACTCTATACT
9541	CAATCAAGCA	AAATTTCTGT	ATATTCCCTG	TGGAATGTAC	CTATGTGAGT	TTTCAAGAAAT
9601	CTCAAAATAC	GTGTTCAAAA	ATTTCTGCTT	TTGCATCTTT	GGGACACCTC	AGAAAACTTA
9661	TTAACAACTG	TGAATATGAG	AAATACAGAA	GAAAATAATA	AGCCCTCTAT	ACATAAATGC
9721	CCAGCACAAT	TCATTGTTAA	AAAACAACCA	AACCTCACAC	TACTGTATTT	CATTATCTGT
9781	ACTGAAAGCA	AATGCTTTGT	GACTATTAAA	TGTTGCACAT	CATTCAATCA	CTGTATAGTA
9841	ATCATTGACT	AAAGCCATTT	GCTGTGTTTT	CTTCTTGTGG	NTGNATATAT	CAGGTAAAAAT

+ Gen Bank
ends

9901 ATTTTCCAAA GAGCCATGTG TCATGTAATA CTGAACCCTT TGATATTGAG ACATTAATTT
9961 GGACCCTTGG TATTATCTAC TAGAATAATG TAATACTGNA GAAATATTGC TCTAATTCTT
10021 TCAAAATGGT GCATCCCCCT TAAAANGTTC TATTTCCATA AGGATTTAGC TTGCTTATCC
10081 CTTCTTATAC CCTAAGATGA AGCTGTTTTT GTGCTCTTTG TTCATCATTG GCCCTCATTG
10141 CAAGCACTTT ACGCTGTCTG TAATGGGATC TATTTTTGCA CTGGAATATC TGAGAATTGC
10201 AAAACTAGAC AAAAGTTTCA CAACAGATTT CTAAGTTAAA TCATTTTCAT TAAAAGGAAA
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10321 TGTTGTAATA TAATGAGTCA CAAAATAAAG CTGTGACAGT TCTGTTAAAA AAAAAAAAAA
10381 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
10441 AA

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TITLE _____

From Page No. _____

Important Results of BLAST search ABCR versus ABC1 (our clone)

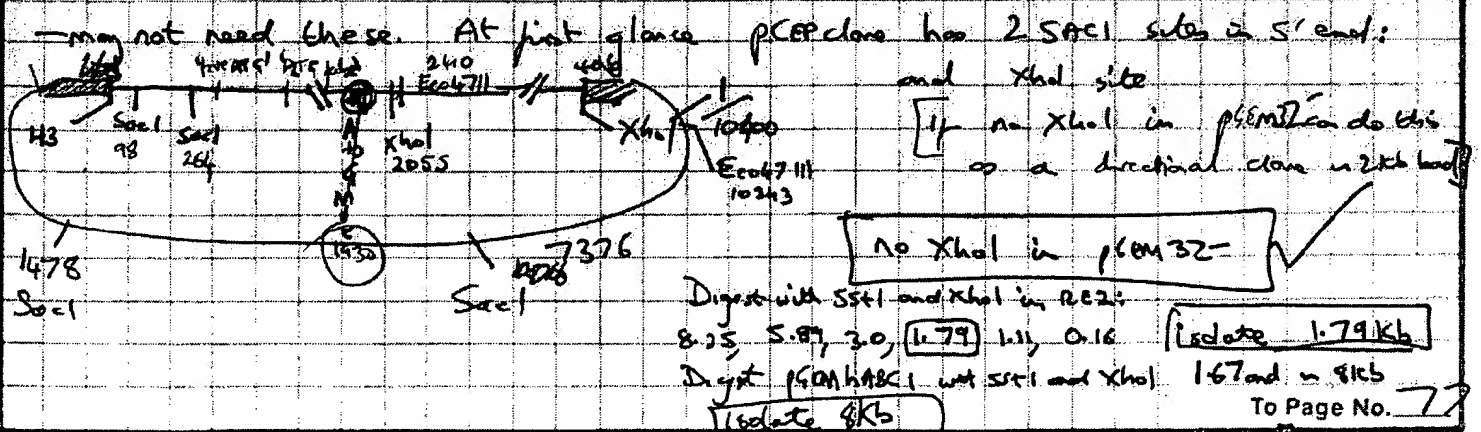
I noticed that the homology between our ABC1 sequence and ABCR started at position 338 on our sequence. But the ^{putative} ATG start (p. ABC1) is at 502. ABCR is very close to ABC1 by homology search, and is 2223 residues long, but ABC1, as given in Chinn's paper, is 2021 aa.

So I truncated rest of 5' region of our clone.

→ There is a possible 60 residue additional Nth terminus on ABC1. It is preceded by a in frame stop codon! suggesting a true translation start site. Plus it looks a bit like a signal peptide!!

Lets the arguement failed to notice this - potential consequences enormous! Exactly the same residues are present in mouse ABC1, though ORF goes back further (no information). So we must ① redo all our constructs to include this.

② Purify enough of the protein to sequence to find real amino terminus.

NEW PRIMERS FOR 3' END OF HABC1

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

The human ABC1 protein contains 60 more amino acids at its amino terminus than reported by other groups

The published protein sequence of human ABC1 protein, based on translated cDNA sequence, claims that the protein begins with the sequence MPSAGTLPWVQGII... (Langmann et al., *Biochim. Biophys. Res. Comm.* **257**, 29-33, 1999; GenBank Accession # AJ012376) and that the protein contains 2201 amino acids. This has been cited and assumed to be correct in all publications about ABC1 to date of which we are aware. This is important because authors have claimed to possess "full length cDNA clones", to design PCR primers to reproduce the full length coding region, to compare the full length coding region in normal individuals and those with diseases of HDL metabolism such as Tangier disease and familial hypo-alpha-lipoproteinemia, and to design expression plasmids to produce recombinant full length ABC1 protein based on this proposition. We believe it to be incorrect for the following reasons:

Our cDNA sequence of human ABC1 predicts an open reading frame that continues from the "Langmann" predicted start methionine, upstream for 60 more amino acids to a methionine codon, predicting that the true amino terminus of human ABC1 is:

MACWPQLRLLLWKNLTFRRRQTCQLLLEVAWPLFIFLLISVRLSYPPYEQHECH
FPNKA.... followed by the previously reported sequence beginning

MPSAGTLPWVQGII... There is an in-frame stop codon 6 to 9 nucleotides upstream from this location, so this would be the first methionine codon which could produce a continuous open reading frame. Alignment of our ABC1 cDNA sequence with GenBank sequences predicts a highly significant degree of similarity with other related ABC sequences, including the ABCR and ABC-C (also known as ABC3) transporters, which are also open reading frames for these 60 amino acids, implying that all three of these homologous proteins begin with sequences related to the amino terminal extension we propose for human ABC1. Presumably, the error in Langmann arose from their reliance on the published cDNA sequence of mouse ABC1 (Luciani et al. *Genomics* **21** 150-159, 1994). There, without direct evidence, they predicted an open reading frame of 2201 amino acids "potentially covering the whole coding region". In fact, they may have been in error in this presumption, because in their cDNA sequence (GenBank Accession

number X75926) there was an uncalled nucleotide "n" in this region, not allowing them to be sure that an open reading frame could have extended further upstream to an additional methionine codon. Following this potentially false lead, Langmann et al. designed PCR primers with which to obtain their human cDNA that did not extend far enough upstream to contain what we now propose to be the true extent of the coding region. In fact, if this undetermined "n" nucleotide in the mouse sequence is ignored, the mouse and human sequences of the "extra" 60 amino terminal amino acids are identical. In light of these results, it is highly likely that the true full length human ABC1 protein contains 2261 amino acids, and not the 2201 amino acids claimed by Langmann et al and in the subsequent publications to date which compare its sequence in normal individuals to those with Tangier disease. Recombinant DNA constructs useful to produce functional ABC1 protein or for gene therapy should contain the entire coding region for 2261 amino acids.

Direct proof of the true amino terminus of ABC1 can be obtained by amino terminal protein sequencing, followed by comparison to the sequence predicted by translation of the cDNA sequence. This can be accomplished by preparing membrane-bound protein preparations of macrophage or fibroblast cells, preferably after induction of ABC1 gene expression by cholesterol loading or 8-Br-cAMP treatment, as we have shown, to increase the yield of the desired protein. Partial purification can be performed by immunoprecipitation with anti-ABC1 antibodies, followed by SDS gel electrophoresis. The stained band corresponding to the molecular weight of 240 kDa is then excised after transfer of proteins from the gel to membrane, and amino terminal sequence determined by standard Edmon degradation procedures.

Richard L. Lacey

David W. Lane

CVTHABC1 PROTEIN 2261 AA

Base pairs 291 to 7076 of CVThABC1 Nucleotide translated.

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1 MACWPQLRLL LWKNLTFRRR QTCQLLLEVA WPLFIFLILI SVRLSYPPYE QHECHFNPKA
61 MPSAGTLPWV QGIICNANNP CFRYPTPGEA PGVVGNFNKS IVARLFSDAR RLLLYSQKDT
121 SMKDMRKVLR TLQQIKKSSS NLKLQDFLVD NETFSGFLYH NLSLPKSTVD KMLRADVILH
181 KVFLQGYQLH LTSLCNGSKS EEMIQLGDQE VSELCGLPKE KLA AAERVLR SNMDILKPIL
241 RTLNSTSPFP SKELAEATKT LLHSLGTLAQ ELFSMRWSW MRQEVMFELTN VNSSSSSTQI
301 YQAVSRIVCG HPEGGGLKIK SLNWDYEDNNY KALFGGNGTE EDAETFYDNS TTPYCNDLMK
361 NLESSPLSRI IWKALKPLL V GKILYTPDTP ATRQVMAEVN KTFQELAVFH DLEGMWEELS
421 PKIWTFMENS QEMDLVRMLL DSRDNDHFWE QQLDGLDWT A QDIVAFLAKH PEDVQSSNGS
481 VYTWREAFNE TNQAIRTISR FMECVNLNKL EPIATEVWLI NKSMELLDER KFWAGIVFTG
541 ITPGSIELPH HVKYKIRMDI DNVERTNKIK DGYWDPGPRA DPFEDMRYVW GGFAYLQD VV
601 EQAIRVLTG TEKKTGVYMQ QMPYPCYVDD IFLRVMSRSM PLFMTLAWIY SVAVIK GIV
661 YEKEARLKET MRIMGLDNSI LWFSWFISL IPLLVSAGLL VVILKLG NLL PYSDPSV VV
721 FLSVFAVVTI LQCFLISTLF SRANLAAACG GIIYFTLYLP YVLCVAWQDY VGFTLKIFAS
781 LLSPVAFGFG CEYFALFEEQ GIGVQWDNLF ESPVEEDGFN LTTSISMMLF DTFLYGVMTW
841 YIEAVFPGQY GIPRPWYFPC TKS YWFGEES DEKSHPGSNQ KRMSEICMEE EPTHLKLGVS
901 IQNLVKVYRD GMKVAVDGLA LNFYEGQITS FLGHNGAGKT TTMSILTGLE PPTSGTAYIL
961 GKDIRSEMST IRQNLGVCPQ HNVLFDM LTV EEHIW FYARL KGLSEKHVKA EMEQMALDVG
1021 LPSSKLKSKT SQLSGGMQRK LSVALAFVGG SKVVILDEPT AGVDPYSRRG IWELL LKYRQ
1081 GRTIILSTHH MDEADV LGDR IAIISHGKLC CVGSSLFLKN QLGTGY YLTL VKKDVESSL S
1141 SCRNSSTVS YLK KEDSVSQ SSSDAGLGSD HESDTLTIDV SAISNLIRKH VSEARLVEDI
1201 GHELT YVLPY EAAKEGAFVE LFHEIDRLS DLGISSYGIS ETTLEE IFLK VAEESGVDAE
1261 YSDGTL PARR NRRAFGDKQS CLRPFTEDDA ADPNDS DIDP ESRETDL LSG MDGKGSYQVK
1321 GWKLTQQQFV ALLWKRL LIA RRSRKGFFAQ IVLPAVFVCI ALVFSLIVPP FGKYP SLELQ
1381 PWMYNEQYTF VSNDAPEDTG TLELLNALT K DPGFGTRCME GNPIPDTPCQ AGE EEWTTAP
1441 VPQTIMDLFQ NGNWTMQNPS PACQCSSDKI KKMLPVCPPG AGGLPPPQRK QNTADILQDL
1501 TGRNISDYL V KTYVQIIAKS LKNKIWVNEF RYGGFSLGVS NTQALPPSQE VND AIKQMKK
1561 HLKLAKDSSA DRFLNSLGRF MTGLDTRNNV KVVFNK GWH AISSFLNVIN NAILRANLQK
1621 GENPSHYGIT AFNHPLNLTK QQLSEVALMT TSVDVLVSIC VIFAMSFVPA SFVVFLIQR
1681 VSKAKHLQFI SGVKPVIYWL SNFVWDMCNY VVPATLVIII FICFQQKSYV SSTNL PVLAL
1741 LLLLYGWSIT PLMYPASFVF KIPSTAYVVL TSVNLFIGIN GSVATFVLEL FTDNKLNNIN
1801 DILKSVFLIF PHFCLGRGLI DMVKNQAMAD ALERFGENRF VSPLSWDLVG RNLFAMAVEG
1861 VVFFLITVLI QYRFFIRPRP VNAKLSPLND EDEDVRRERQ RILDGGGQND ILEIKELTKI
1921 YRRKRKPAVD RICVGIPPE CFGLLG VNGA GKSSTFKMLT GDTTVTRGDA FLNKNSILSN
1981 IHEVHQNMGY CPQFDAITEL LTGREHVEFF ALLRGVPEKE VGKVGEWAIR KLGLVKYGEK
2041 YAGNYSGGNK RKLSTAMALI GGPPVVF LDE PTTGMDPKAR RFLWNCALSV VKEGRSVVLT
2101 SHSMEECEAL CTRMAIMVNG RFRCLGSVQH LKNRFGDGYT IVVRIAGSNP DLKPVQDFFG
2161 LAFPGSVLKE KHRNMLQYQL PSSLSLARI FSILSQSKKR LHIEDYSVSQ TTL DQVFVN F
2221 AKDQSDDDHL KDLSLHKNQT VVDVAVLTSF LQDEKVKESY V*
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